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Biopolymer Local Delivery Device Loaded with Rifampin and Ciprofloxacin to Inhibit Biofilm Formation

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BIOPOLYMER LOCAL DELIVERY DEVICE LOADED WITH RIFAMPIN AND CIPROFLOXACIN TO INHIBIT BIOFILM FORMATION

by

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DEDICATION

I would like to dedicate this thesis to my loving and supportive family who have been there, whenever needed, every step of the way. Their display of love, constant encouragement, motivation, and invaluable knowledge allowed me to persevere through and continue to strive towards my goals in academia and life.

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PREFACE

The main body of this thesis in Chapter III is a journal article entitled “Ciprofloxacin and Rifampin Dual Antibiotic-Loaded Biopolymer Chitosan Sponge for Bacterial Inhibition.” This manuscript was submitted as an invited supplement to the Journal of Military Medicine.

ABSTRACT

Increases in patients' costs, multiple surgeries, and difficult treatment solutions are resultants of musculoskeletal infections. The presence of biofilm-forming or antibiotic resistant bacteria exponentially increase the complexity and complications in treatment of those musculoskeletal infections. During this study, the combination of ciprofloxacin and rifampin loaded in and released from chitosan-based local delivery systems was evaluated as an adjuvant therapy for prompt reduction of biofilm-forming bacteria in the wound when locally delivered. Primary assessments included antibiotic release, sponge eluate *in vitro* activity, *in vitro* synergy assays, effect on chitosan sponge pore structure, and an *in vivo* implant associated biofilm functional model. Antibiotic activity was present through seven days against *S. aureus* and *P. aeruginosa*. Ciprofloxacin had a therapeutic elution profile that lasted, at least, seven days while rifampin's lasted three days. Additive effects were present against *P. aeruginosa* during the *in vitro* synergy assay with inconclusive results against *S. aureus*. No unexpected or adverse effects on chitosan sponge pore structure were seen after sponges were loaded with the antibiotic cocktail. Complete clearance of biofilm-forming *S. aureus* and *E. coli* with no noticeable adverse effects were achieved in the functional infected pin murine model. The results of this study support the potential use of ciprofloxacin and rifampin in chitosan-based and other local delivery devices, as local adjunctive therapy, for prevention of musculoskeletal and surgical site infections.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Statement of Clinical Problem

Of all injuries in the United States one of the more prevalent types are complex musculoskeletal wounds, which may experience compromised wound healing as a result of bacterial infection.[1] Civilians are estimated to have a 20% chance of infection in open fracture wounds with soldiers having estimates up to 65% if an open fracture wound is the result of a high-energy trauma.[1-3] Bone fixation devices may be required to ameliorate traumatic injuries to the musculoskeletal system. According to estimates, incidences of open fracture and extremity trauma are estimated to be six million or more each year in the United States.[4] Infection rates of 5% are seen when fixation devices are implemented in musculoskeletal injuries.[5] Recently released estimates from the American Academy of Orthopedic Surgeons (AAOS) reflect over 500,000 surgical site infections (SSI) occurring per year subsequent to an orthopedic procedure.[6] It was reported between October 2005 – December that 14.8% of total hip arthroplasty (THA) revisions were due to infection.[7] Occurrence of bacterial resistance is increasing, magnifying an already difficult treatment prospect of musculoskeletal infections.[6] Adverse effects may result from an increase in systemic antibiotic administration.[8] Some current solutions such as poly(methyl methacrylate) beads offer minimal to no degradation, requiring an additional surgery for removal.[9, 10] Treatment solutions incorporating calcium sulfate may experience the onset of excessive fluid accumulation and wound drainage resulting from rapid degradation.[11] Engineered inexpensive biodegradable devices, loaded with widely available antibiotics, could be advantageous to reduce or prevent SSIs in orthopaedic procedures and especially in

conjunction with the regimen aimed at complex extremity wounds coalesced with fracture fixation devices.

Hypothesis and Research Objectives

It was hypothesized that engineered porous chitosan sponges, after being hydrated with ciprofloxacin and rifampin for short-term treatment, less than 14 days, will be effective as an adjunctive therapy for infection inhibition. Other goals include maintaining elution kinetics to maintain diffusion of active antibiotics to inhibit establishment of bacterial infections in musculoskeletal injuries, cytocompatibility, and capability of point-of-care loading. The specific objectives of this research are as follows:

1. Maintain bactericidal activity at or above minimal inhibitory concentrations against biofilm-forming *P. aeruginosa* and *S. aureus* of ciprofloxacin and rifampin after being eluted from chitosan-based local delivery device
2. Possess biodegradable characteristics in chitosan-based sponges that will eliminate need of additional surgery for removal, after application, of local delivery device
3. Possess cytocompatibility/biocompatibility properties of chitosan-based local delivery device that support use in infection prevention applications
4. *In vitro* delivery of ciprofloxacin and rifampin from the chitosan-based sponges sustained through, at least, 7 days at active levels against biofilm-forming *S. aureus* and *P. aeruginosa* to prevent colonization
5. *In vivo* delivery of ciprofloxacin and rifampin from chitosan-based sponges over, at minimum, 7 days for inhibition of bacterial infection resulting from biofilm-forming *S. aureus* and *E. coli* in a functional mouse pin model

CHAPTER II

LITERATURE REVIEW

Wounds and Healing Process

Musculoskeletal wound infections may develop from traumatic injuries, surgical wounds, acute wounds lacking proper treatment, or from an accompanying co-morbidity; and may prevent normal patient healing and recovery.[12] Peripheral vascular disease, diabetes, malnutrition, and chronic steroid use are some co-morbidities which can impair tissue healing and contribute to the occurrence of surgical site and wound infections.[13] High-energy fractures where the bone penetrates the skin, compromises vasculature and communicates with extra-tissue environment—open fracture—is an extreme case of traumatic musculoskeletal wound.[14] Open fractures are more likely to establish bacterial colonization when compared to surgically created wounds.[15] Emotional, physical, and monetary stress experienced by the patient resulting from musculoskeletal wounds are also high.[7]

Impairment of normal physiology resulting from damage to muscle, skin, bone, or other tissue is referred to as a wound.[16] Innate responses in efforts to repair the wound occurs in a series of six steps normally. Rapid hemostasis is primary after a wound is initiated, which is the mechanism that stops the bleeding.[16] Vasoconstriction, tight closing of blood vessels, of wound feeding vessels and clotting are the body's mechanisms normally used to achieve this goal.[16] Inflammation is the body's way to signify an injury it is secondary/simultaneous to hemostasis. Healthy cells are being directed to the wound site during the process of inflammation and is one reason why inflammation is vital in the wound care process.[16] However, inflammation can be detrimental to wound healing by preventing regeneration if it prolongs. There are four primary symptoms associated with inflammation, redness, swelling, heat, and pain. As inflammation is

occurring several types of cells are being released including ones for migration and proliferation. Movement of cells in an extremely coordinated and specific order happens with migration function. Further constriction of blood vessels, like hemostasis, happens under proliferation. As bleeding becomes controlled the body begins engaging in rebuilding tissue. The formation of new blood vessels during a process referred to as angiogenesis is the primary step in the rebuilding process. Damaged veins and arteries are beginning to be replaced through either creation of new sections or with addition to existing sections. After angiogenesis is the replacement of compromised skin, known as re-epithelialization. Keratinocytes, which comprise the epidermis, are proliferating and migrating to cover the wound from the environment during this phase.[16] During this phase, several layers are created each working in tandem to prevent loss of fluid and provide protection.[16] These are innate would healing processes that most humans possess and employ if a wound is experienced. There are several instances when the innate processes may be overwhelmed and require an assist, some musculoskeletal wound infections are a prime example.

Various cells types have specific roles in hemostasis, inflammation, proliferation, and remodeling, which are aspects of innate wound healing.[17] Neutrophils migrate from the blood to remove foreign bodies, bacteria, non-viable tissue and other debris from the wound site within the first eight hours.[18] Maximal concentration of neutrophils at the site of the wound is reported to occur in 24 – 48 h with decreased concentration beginning 72 h after wound induction.[19] Macrophages and mast cells become responsible for the cleansing process as neutrophils evacuate the wound site.[20] Epithelial cells construct a thin epithelial layer over the wound, closing it from the external environment after the wound is clean and disinfected, which occurs approximately after 48 – 72 h.[21, 22] Subsequent to the cellular/tissue responses discussed

previously, remodeling is initiated and persists, on average, 4 – 8 weeks depending on the severity of the wound.[23]

Wound Management

Effective management of the wound can assist the patient's innate response in restoration of function to the wound site. A reduction in morbidity with improved prognosis is seen through the prevention of complications in the healing process.[24] Debridement, irrigation, fixation, closure and infection prevention are main principles of complex musculoskeletal wound management.[25] Currently, no universal standard exists in application of these principles by surgeons.[26]

The process of debridement removes foreign and non-viable tissue from the wound.[27] Necrotic tissue is the ultimate product of non-viable tissue and inhibits wound healing with the promotion of microbe growth and the blockage of the host's inherent tissue defenses.[27] As a result, debridement of skin and subcutaneous tissue is executed until capillary bleeding is visualized.[26] Additional debridement may be recommended after 24 – 48 h depending on the degree of soft tissue damage or contamination.[28]

Removal of smaller foreign bodies coupled with the reduction in bacterial concentration is known as irrigation or lavage. While there is some variation in irrigation procedures, in general copious amounts (approximately 10 L) of irrigation fluid are used to help remove small foreign bodies and bacteria from wound site.[28] When ballistic or explosion wounds are present irrigation is essential due to the potential for the bacteria to spread along tissue planes.[29] In efforts to minimize patient infection, antimicrobial administration by means of irrigation also may be implemented.[30] The knowledge level of the presiding surgeon regarding the wound

affords them the opportunity to tailor the antibiotic lavage treatment for specific suspected bacterial contaminate.[31]

Further bone and soft tissue damage is inhibited by wound fixation and closure, which provides an initiation point for inherent wound healing procedures.[32] Several methods for internal fracture stabilization exist including, but not limited to, intramedullary nails, plates and screws with each possessing pros and cons.[33] Intramedullary nails are effective when fixation of lower extremity fractures are sought, however there are possibilities that bone circulation may be disrupted.[34, 35] Plates and screws are often employed when precision is needed for a fractured bone alignment and are mostly used in upper extremity and transarticular fractures.[36, 37] For more advanced wounds entailing crushed or pulverized bone and surrounding soft tissue damage, treatment solutions may include internal fixation devices such as rods and scaffolds composed from titanium or stainless steel alloys or external devices consisting of frame and pins.[38, 39] When considering prosthesis associated with surgical procedures performed during total hip and total knee arthroplasties material fixation becomes critical.[40] Closure of wounds may be achieved with available soft tissues, depending on whether adequate coverage is possible, or soft tissue reconstruction from local or free muscle flaps.[32] The surgeon may, ultimately, be constricted to amputation due to damage or infection.[28]

Wound Infection

Prevention is often the most desired goal in infection treatment, however approximately 65% of patients with severe, open wounds have microorganism contamination.[2]

Among the six million fractures occurring every year, 5 – 10% will entail additional treatment for compromised healing resulting from improper fracture fixation, bone resorption, vascular impairment or infection. Infection risks are increased if co-morbidities preexist and once

infection is established symptoms may include fever, chills, headache and stiffness to pain, erythema and wound drainage.[2] Imaging, microbiological cultures, blood tests, and polymerase chain reaction (PCR) techniques are infection diagnostic tools.[41-43] Systemic antibacterial therapy is a technique that does not involve surgery as an initial treatment.[44] Long term effects of wound infections include revision surgeries, continued antibiotic treatments, possible recurrence of infection, prolonged wound healing and reduced quality of life, which all contribute to increased medical costs.[42] Potential clinical outcomes include patient death or treatment failure.[45, 46] Within the United States surgical site infections (SSI) are accountable for an approximate $1.6 billion inflation in healthcare costs per year.[47] Estimates for average attributable costs of SSIs include estimates ranging from $10,443 (2005 dollars)[48] to $25,546 (2002 dollars)[49] per infection. According to a Centers for Disease Control and Prevention report by Scott *et al*., the infection site with the largest range of annual costs is SSI $3.2 billion to $8.6 billion using the Consumer Price Index for all urban consumers (CPI-U) and $3.5 billion to $10 billion using the Consumer Price Index (CPI) for inpatient hospital services.[50] The CPI-U is constructed by the U.S Bureau of Labor Statistics and is a measure of the average change over time in prices paid by all urban consumers (defined as all urban households in Metropolitan Statistical Areas and in urban places of 2500 inhabitants or more) for a market basket of consumer goods and services purchased for day-to-day living.[50]

Staphylococcus aureus (S. aureus), Staphylococcus epidermidis (S. epidermidis) and *Pseudomonas aeruginosa (P. aeruginosa)* are common in musculoskeletal wounds.[2] Often isolated from the skin and gastrointestinal (GI) tract of healthy individuals' flora with the ability to cause disease when introduced to wound sites.[2] *S. aureus* is commonly found in musculoskeletal infections consisting of osteomyelitis, arthritis, myositis, or implanted

orthopaedic devices and arises in roughly 50% of all infections.[2, 51] *S. aureus* is reported as the leading cause of osteomyelitis and nongonococcal bacterial arthritis[52] and a prominent cause of prosthetic joint infection.[2, 5] Escalation of the problem was seen in the 90's as *S. aureus* began to exhibit resistance to certain antibiotics.[45] *S. aureus* resistance nullifies the effectiveness of many β-lactam antibiotics, including methicillin specific antibiotics, which act to inhibit Gram-positive bacteria's peptidoglycan cell wall synthesis.[42] Methicillin resistant *Staphylococcus aureus* (MRSA) infections have seen an increase from 35.9% in 1992 to 64.4% in 2003 for *S. aureus* isolates collected in the United States.[45, 46] The American Academy for Orthopedic Research recently indicated the death rate from MRSA is 2.5 times greater than its non-resistant counterpart.[5] Propagation of vancomycin resistant *S. aureus* (VRSA) is a grave concern.[53, 54] The resistance to vancomycin by *S. aureus* has seen increasing importance in nosocomial pathogens in the United States, but to date has seen little occurrence in osteomyelitis.[55]

The commonly reported infection rate through prophylactic antibiotics and aseptic techniques for uncontaminated surgical wounds is 1%, however for open fractures the rate is approximately 50%.[12] A wide-spectrum of Gram-positive and Gram-negative specific antimicrobial agents are commonly administered to open fracture wounds to prevent contamination. Bacteria may adhere to wound tissue and on implanted biomaterial surfaces subsequently forming an enclosed film of polysaccharides, ions, nutrients, and other environmental constituents as a mechanism of survival.[56] Adhered bacteria are known as biofilm. According to reports the increase in biofilm related bacterial resistance is amplified by the increase in betalactamase concentration within the biofilm and the inability of antibiotic to penetrate biofilm.[5] The presence of implant devices strengthens the likelihood of infection and biofilm, making implant materials an infection risk factor.[56, 57]

Implant Associated Infection

Host defense weaknesses that allow bacteria to become established are exploited in orthopaedics with implant concomitant post-surgical infections.[56] Currently, simple debridement and irrigation procedures allied with antimicrobial agents are not always efficacious in prevention of infection specifically with open fractures, which have an infection rate of 50%.[14, 58] Due to the large population of patients receiving orthopaedic implants with an infection rate of 1 - 5% for those involving total joint replacement, infections related to implants are considered prime for novel research in infection prevention treatments.[5, 7, 56] Often, additional surgeries for implant removal and replacement are the only option for infection eradication. Bacteria are passively or actively adsorbed after implantation on biomaterial surfaces leading to biofilm formation. One fundamental pathogenic mechanism in biomaterial-centered sepsis is microbial colonization followed by adjacent tissue damage.[56] Main pathogenic species of orthopaedic clinical isolates related to infections linked to implants are 34% *S. aureus*, 32% *S. epi*, 13% coagulase negative *staphylococci*, 6% *pseudomonas*, 5% *enterococcus*, and 11% other species.[2] A vast majority of infections associated with implants in orthopaedics are the result of Gram-positive aerobes, primarily *staphylococci*.[6]

Increases in morbidity, mortality and socioeconomic costs can be attributed to biomaterial-centered infections.[47] Preventing infection formation is an ongoing and growing challenge to implant development.[28, 56] Areas for improvement include more efficacious antibiotic treatment of implant associated infections and traumatic musculoskeletal wounds.

Antibiotic Therapy

Selection of antibiotics for the treatment of musculoskeletal infection may be distinguished by three phases: 1st visual, 2nd definitive treatment, and 3rd treatment

confirmation.[59] The risk of infection in patients with severe wounds is reduced with early antibiotic administration.[44] The selection of antibiotics by the attending physician or clinician is constrained by their ability to target the bacteria commonly seen in infections that the patients are deemed to be at risk for. Increases in the occurrence of antibacterial resistance magnifies the arduous task of combating musculoskeletal wound infections.[44] Due to recent advancements, wound screening has the ability to identify the presence of certain microbes prior to treatment.[6, 41] With early dispensation of selected antibiotic therapy the effectiveness has improved, aiding in limiting exposure and preventing infection.[59]

Quinolones/fluoroquinolones have proven to be effective against Gram-negative and some Gram-positive bacteria due to their ability to prevent the DNA from unwinding and duplicating.[60] Principally, quinolones/fluoroquinolones target DNA gyrase and topoisomerase IV, which are both bacterial type II topoisomerases.[61] DNA gyrase is a tetramer enzyme with two GyrA and two GyrB subunits that catalyze the introduction of negative superficial turns into, and elimination of positive supercoils from, the replication fork into the double-stranded covalently closed circular DNA.[62] DNA gyrase acts before the replication fork, preventing replication-induced structural changes.[63] Topoisomerase IV acts behind the replicating fork and catalyzes the removal of supercoils and decantation of interlocked daughter DNA molecules.[64] Both DNA gyrase and topoisomerase IV appear to be targeted by all quinolones, regardless of bacterial species.[62] In Gram-negative bacteria, DNA gyrase is generally bound more readily by quinolones.[62] Topoisomerase IV is preferred in Gram-positive bacteria.[65, 66] Although this effect on bacteria by quinolones has proven useful and have successfully prevented the duplication of certain bacteria, quinolones alone have proven ineffective, comparable to other classes of antibiotics, against biofilm when used alone.[67-71] Ciprofloxacin is a 2nd generation

fluoroquinolone that has been clinically effective at inhibiting Gram-negative and some Gram-positive bacteria.[67-76] Second-generation quinolones are *fluorinated* quinolones discovered in the mid-1980s.[62] The fluorine atom and piperdine positioning result in a much broader spectrum of activity that includes *P. aeruginosa* and some Gram-positive bacteria, such as *S. aureus*, and improved bioactivity.[62] While ciprofloxacin has shown the ability to penetrate biofilm, bacterial resistance has also been developed.[77] Ciprofloxacin is one of the numerous fluoroquinolones that have extensive clinical usage for the treatment of Gram-negative and some Gram-positive bacterial pathogens.[63, 65, 66, 78-83] Potential acquired resistance to ciprofloxacin, along with massive list of other antibiotics, presents challenges that require novel approaches.[60, 67, 68, 84-95] Among these novel approaches is the usage of antibiotics in combinations that may facilitate possible synergism of each antibiotic's active mechanism against polymicrobials. However, systemic administration of ciprofloxacin has recently been noted to cause adverse side-effects involving tendons, muscles, joints, nerves and the central nervous system by the FDA.[96]

Rifampin has emerged as a major player in antibiotic combination therapy for its ability to combat biofilm among other infections. Rifampin, a type of rifamycin, specifically inhibits bacterial DNA-dependent RNA polymerase by blocking the RNA chain initiation step of bacterial DNA transcription to messenger RNA that is needed to synthesize bacterial proteins.[97] Rifampin is employed as part of combination regimens against mycobacterial infections and some Gram-positive bacterial infections, such as *S. aureus* and *S. epidermis*.[62] Rifampin is bactericidal against Gram-positive bacteria and diffuses well into biofilm where it perpetuates its bactericidal ability.[62] Disruption of biofilm is within the scope of rifampin, however rifampin-resistant mutants may be selected.[62] Rifampin requires administration in conjunction with other antibiotics as a result.[62] Prior *in vitro* studies have demonstrated rapid synergy when rifampin is

incorporated with a β-lactam and slower synergy with vancomycin.[89, 97, 98] Synergy is defined in the medical dictionary as the correlated action or cooperation by two or more structures or drugs. Synergism is the state at which the combined action is greater than the sum of each acting separately. Rifamycins bind very tightly to the β-subunit of the RNA polymerase in a molar ratio 1:1.[62] In contrast they do not bind well to the β-subunits isolated from rifamycin-resistant mutants and mutations are found in the β-subunits of RNA polymerase in such mutants that aid in a reduction of rifampin binding to β-subunits of RNA polymerase.[62] There is currently no evidence to support an effect on mammalian enzymes by rifampin.[62] Rifamycins also modify bacterial pathogenicity and may alter attachment and tissue toxicity.[99-101] The World Health Organization's List of Essential Medicines includes rifampin and ciprofloxacin. Multiple investigations are ongoing consisting of rifampin in combination with other infection reducing agents.[88, 89, 102-116] Resistance to rifampin, despite its contrivance, has been demonstrated to develop quickly.[56, 117-120] Monotherapy is rarely incorporated as a result.[56, 117-120] Due to their methods of action, antibiotics must be in contact with their bacterial targets to be effective.[121, 122] Some antibiotics may be delivered to the infection site either systemically or locally, while some are delegated to being delivered locally.[123]

Localized Drug Delivery

Drug delivery using intravenous or oral routes is well established, however those methods may possess undesired effects related to the distance from the injection site or digestive system; in addition to the biochemical environment that must be navigated before the drug reaches the wound.[124] The success of antibiotic therapy depends on keeping low, nontoxic levels systemically and high levels in the infection tissue.[8] Local therapy using antibiotic-loaded delivery vehicles have been utilized adjunctive to systemic antibiotic therapy for open wound

and fracture treatments. Antibiotic release from the delivery mechanism into the surrounding tissue is referred to as elution, which is facilitated by increased surface area/volume ratio and high concentrations of loaded antibiotics.[124] Protracted release of drugs, which may lead to high local levels of bioactive antibiotics with negligible serum levels are obtainable using this technique.[125] Bone penetration is not actively observed with systemically administered antimicrobial agents, in contrast high local levels of antibiotics enable antibiotic delivery through diffusion to bone and avascular areas of wounds.[8] Through the utilization of local drug delivery potency is maximized at infection site, while minimizing systemic toxicity risk generating an efficacious delivery system.[10]

Efficacy of local drug delivery may be improved with the insertion of local biodegradable delivery devices.[126] Biodegradable materials are broken down with hydrolytic mechanisms with or without supplemental enzymatic mechanisms. A primary advantage of biodegradable systems is the circumvention of subsequent surgeries to remove the foreign drug delivery device. Implantation at the site of the wound allows the drug to be delivered at amplified concentrations with minimal delay between delivery and action, with degradation occurring naturally over time. The reported status of local drug delivery in an article by McLaren, stated that all are pre-loaded with antibiotics during the manufacturing process, referred to PMMA and calcium sulfate (CaS).[10] McLaren concluded that no polymer was easily mixed with antibiotics in the operating room or *in situ*-loaded for clinical use.[10] *In situ*-loading would offer the advantage of being implemented locally at the site of the wound or infection at the time of treatment, which chitosan sponge based local delivery systems offer.

Chitosan

Chitosan may be used to fabricate local biodegradable delivery devices. Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-2-amino-2-D-glucosamine (deacetylated) and β-(1-4)-2-acetamido-2-D-glucoseamine (acetylated) units.[127] The second most abundant naturally occurring polymer, chitin, is used to derive chitosan.[128] Chitin is a white, hard and inelastic nitrogenous polysaccharide that is isolated from exoskeletons of crustaceans. Strong alkaline solution is the major procedure used for obtaining chitosan by alkaline deacetylation of chitin.[129] The raw material is crushed, washed with water or detergent and ground into small pieces prior to processing. The abundance of acetyl units is the chemical difference between chitosan and chitin. Chitosan's performance is impacted by its degree of deacetylation (DDA).[130, 131] When the DDA is greater than 50% the copolymer is termed chitosan. Chitosan possesses a weak cationic base, is insoluble in water and organic solvents but is soluble in dilute acidic solutions, which may include acetic, citric, formic or lactic acid.[130, 132] Negatively charged surfaces are bound by chitosan due to bioadhesive properties resulting from its charges (NH_3^+).[133] Chitosan is characterized as biodegradable,[128, 134, 135] antibacterial[127, 136] and possesses ability to be loaded with and release drugs over time.[124, 136-138]

The level of chitosan's DDA affects degradability with DDA close to 50% being highly degradable *in vivo*, however when DDA is greater than 95% chitosan may remain *in vivo* for months even years.[139] Lysozyme, N-acetyl-o-glucosamindase, and lipases degrade chitosan within the body. Lysozyme degrades chitosan by cleaving the glycosidic bonds between the repeating units, with byproducts of saccharides and glucosamines which are incorporated into glycoproteins or excreted as carbon dioxide.[140] Chitosan's saccharide degradation products do not elicit chronic foreign body reactions because of gradual absorbtion by the human body.[128] In

contrast, when chitosan is more crystalline, normally due to high DDA, the chains are closely packed averting lysozyme interaction which reduces degradation capacity.[139] Implementation of chitosan in a biodegradable local delivery system for antibiotics obviates the need for a removal surgery, reducing the total cost and trauma to the patient in comparison to other local antibiotic delivery devices such as polymethylmethacrylate (PMMA) beads.[128]

Over 200 applications incorporating chitin, chitosan or a derivative are being investigated or in practice.[127, 129] Cosmetics, agriculture, food, biomedical and textile industries are a few examples of the applications. For medical uses, chitosan may be used as a component of wound dressings,[141, 142] bioactive coatings for orthopaedic and craniofacial implants [58, 143] and drug delivery systems amongst many others.[144-150] Chitosan-based drug delivery systems have been loaded with several types of drug molecules, including vancomycin[146], amikacin[149], gentamicin[145], daptomycin[149], prednisolone[148], rhBMP-2[151], rifampin[152], ciprofloxacin.[153] Chitosan has the ability to rapidly clot blood when partially dehydrated, which enables its usage as a bandage or hemostatic agent.[145] Fully hydrated chitosan contains properties that allows it to rapidly rehydrate and absorb drugs.[141]

Biofilm

The formation of biofilm, surface-adhering bacteria, presents challenges that are either costly or exceptionally difficult to overcome in wound treatment, particularly musculoskeletal wounds.[95] Once the formation of the biofilm is detected effectual eradication has proven to be arduous. Biofilm treatment hurdles include the direct communication between the bacteria through the elaboration and recognition of small molecules.[154] *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial strains have been discovered to be among the most common culprits in biofilm.[74, 94] Various antibiotics that possess high levels of activity against many

planktonic bacteria have been evaluated against those same bacteria in a biofilm with little to no success of inhibition. Some studies have demonstrated the ability of several antibiotics to infiltrate bacteria and prevent further growth, however the most effective classes of those same antibiotics against biofilm have revealed bacterial recurrence when used singularly.[114, 155, 156] Reduction in antibiotic vulnerability in the biofilm bacteria is a key component of the potential failure to inhibit or the recurrence.[155]

Biofilm infections that are related to implant devices are problematic because the bacteria has the ability to adhere and form the biofilm on these foreign materials.[91] Microbial colonization has been implicated as the main causative factor in the pathogenesis of implant failures.[157-159] Treatment of implant related biofilm, if unsuccessful, might require additional surgeries to remove the infected tissue or implant component.[160, 161] Developing a treatment method that reduces the required removal of implants due to the formation of biofilm is immensely critical. Patient comfort, costs to patient and/or insurance company (private, company-sponsored or public) and additional potential complications are all concerns once the implant-based biofilm requires removal for adequate management.[50, 56]

Summary

Domestically, infected musculoskeletal wound treatments present challenges that appear perpetual with as much ongoing research in efforts to combat them. The current methods of wound debridement, lavage, and fixation are not able to prevent all infections when instigated solitarily. Infection prevention and wound promotion within the first 72 – 168 hours are crucial objectives that should be met.[32] An increase in antibiotic resistant organisms coupled with the increasing number of surgical site infections are two rising concerns.[43, 45] Rifampin is a viable candidate for the treatment of Gram-positive and resistant *S. aureus* when used in conjunction

with another antibiotic.[74, 116, 162-164] Ciprofloxacin has shown some success against Gram-negative bacteria while being investigated for various applications, making it a feasible aspirant.[72, 74, 165, 166] Local delivery of antibiotics with a biodegradable chitosan sponge would be advantageous for adjunctive musculoskeletal wound treatment. The aim of this research is to (1) determine if the combination of ciprofloxacin and rifampin is efficacious in inhibiting the bacteria known to be present in biofilm when delivered from a chitosan-based delivery device, (2) allow the surgeon or medical provider to load this antibiotic cocktail *in situ*, and (3) sustain antibiotic elution and degradation properties to be applicable as a surgically adaptable device wrap or for wound coverage.

Hypothesis

It is hypothesized that engineered porous chitosan sponges, after being hydrated with ciprofloxacin and rifampin as a short-term adjunctive treatment, less than 14 days, will be effective at biofilm-related infection prevention when situated on or around implanted devices or on traumatic wounds. The sponge will elute antibiotics while biodegrading to inhibit infection, which may develop into biofilm, thus eliminating surgical need for removal of local delivery device.

CHAPTER III

Ciprofloxacin and Rifampin Dual Antibiotic-Loaded Biopolymer Chitosan Sponge for Bacterial Inhibition

ABSTRACT

Complex extremity wounds in Wounded Warriors can become contaminated with microbes, which may lead to amputation, morbidity, or even fatality. Local delivery of multiple or broad-spectrum antibiotics allows practicing clinicians treatment solutions that may inhibit implant-associated infection. The development of vancomycin-resistant *S. aureus* (VRSA) has become a critical challenge in nosocomial infection prevention in the United States. As an alternative to vancomycin in combination with another antibiotic, locally delivered ciprofloxacin and rifampin were investigated in a preclinical model for the prevention of biofilm in complex extremity wounds with implanted fixation device. *In vitro* assays demonstrated ciprofloxacin and rifampin possess an additive effect against Gram-negative *P. aeruginosa* and were actively eluted from a chitosan sponge based local delivery system. In an *in vivo* orthopaedic hardware-associated polymicrobial model (*S. aureus* and *E. coli*) the combination could achieve complete clearance of both bacterial strains. *E. coli* was detected in bone of untreated animals, but did not form biofilm on wires. Results reveal the clinical potential of antibiotic-loaded chitosan sponges to inhibit infection through tailored antibiotic selection at desired concentrations with efficacy towards biofilm inhibition.

INTRODUCTION

Bone fixation devices may be required to ameliorate traumatic injuries and some procedures to the musculoskeletal system. According to estimates, open fracture and extremity trauma are estimated to be six million or more each year in the United States (US).[4] Infection

rates of 5% are seen when fixation devices are implemented in musculoskeletal injuries.[5] Wound infection remains a major cause of morbidity and mortality in US military Service members who experience nonfatal combat-related injuries, notwithstanding current innovations in medical treatment options.[167] Wound infection studies of blast injury resulting from combat operations report several contributing factors, including but not limited to, pathogen, injury type and severity, study population, infection outcome, and geographic zones. These studies reveal that US military personnel have combat-related infection rates ranging from 5.5% to 49%.[168] Multiple factors may contribute to development of musculoskeletal wound infections, including but not limited to, traumatic injuries, surgical wounds, delayed definitive treatment method of acute wounds, or accompanying co-morbidities, which impedes the innate healing process and patient recovery. Rate of occurrence of bacterial resistance to antibiotics is increasing for musculoskeletal infections, magnifying an already difficult treatment prospect.[6] Socioeconomic costs have increased due to the contribution of complications resulting from infection.[28] Engineered inexpensive biodegradable local delivery devices, loaded with widely available antibiotics, could be advantageous to prevent infection, especially infections resulting from biofilm formation on fracture fixation devices. [169-172] More than 65% of infections being treated in the developed world are caused by biofilm forming bacteria.[173]

Injuries that result from combat-related blasts possess a high probability of infection due to the complexity of the wounds and the existence of environmental contaminants, with some studies showing biofilm forming within a matter of hours after injury.[174] Injuries received on the battlefield are further complicated when *Staphylococcus aureus (S. aureus*) and *Pseudomonas aeruginosa (P. aeruginosa*) are involved. These bacteria are the most prevalent Gram-positive and Gram-negative pathogens and are attributed with 75% of biofilm infections on medical

devices.[174, 175] *Escherichia coli* (*E. coli*) and *P. aeruginosa* were two of the more frequent bacterial strains reported during recent military operations, with verified presence in 14% of wounds.[175] Delay of return to duty, an increase in hospitalization associated costs, and/or permanent incapacitation are sequelae that may result from the treatment of infections.[161, 175-177]

Infection prevention after an injury is a principal strategy in managing combat-related trauma, with administration of systemic antibiotics within three hours of injury to reduce the chance of wound infection and bacterial colonization being the primary care option utilized.[174, 176] Local delivery systems for antibiotics, such as antibiotic-loaded poly(methyl methacrylate) beads (PMMA) and calcium sulfate, are growing in acceptance as an adjunctive approach to systemic delivery.[178-180] Local delivery systems may be advantageous over systemic administration in that they deliver higher concentrations of antibiotic directly to the affected tissue[178] and minimize risk of toxicity to organs.[179, 181-183] PMMA beads are non-degradable and require an additional surgery to remove the implanted material for complete wound healing. Additionally, once PMMA beads have released their antibiotic load, they provide surfaces for potential biofilm formation.[184] Rapid resorption of calcium sulfate pellets results in calcium-rich fluid that incites an inflammatory response and wound drainage.[11] The ability to load a local delivery device with broad spectrum or multiple antibiotics by the attending clinician at the time of intervention may reduce the incidence of polymicrobial contamination. Local antibiotic delivery systems should possess the capability for an extended release profile, biocompatibility, biodegradability with biocompatible degradation products, and personalized antimicrobial selection depending on the individual and what bacteria are being targeted.[185]

Chitosan, an adaptable natural biopolymer, has been used to fabricate a myriad of drug delivery systems,[185-187] including lyophilized sponges.[188] Commercially available chitosan

wound dressings in the form of a sponge were approved by the FDA for wound management, possessing the ability to be hydrated with solutions for applications involving varying degrees of musculoskeletal wounds. Practicing physicians, at their clinical discretion, may choose to include antibiotics in the hydrating solution in their treatment solution of contaminated or infected traumatic injuries.[189, 190] FDA clearance of antibiotic introduction into the hydrating solution has not been specifically obtained in any approved local delivery device including PMMA and calcium sulfate. Chitosan sponges have shown excellent biocompatibility, degradability, and may be loaded with countless antibiotics by inert absorption and diffusion.[170, 185, 188, 190, 191] Sponge fabrication allows for varying thicknesses and pore sizes using a lyophilization process, permitting the release of antibiotic solutions to be tailorable,[188, 192] with geometry that can be customized for treatment of complex wounds. This study seeks to investigate the effects of combining ciprofloxacin (Cipro) and rifampin (Rif) for local delivery from chitosan-based sponges for infection prevention. *In vitro* and *in vivo* tests were used to evaluate the efficacy of combined therapy of ciprofloxacin and rifampin against pathogens commonly associated with infection resulting from complex musculoskeletal wounds and/or implants.

MATERIALS AND METHODS

A sample size of 5 allows for detection of a difference of 6 $\mu g\ mL^{-1}$ with an estimated standard deviation of 2.5 $\mu g\ mL^{-1}$ in elution studies with power 0.80. The analysis was based on elution data obtained from previous studies conducted within the lab. This sample size was used throughout the study in all phases, except the *in vivo* animal model (n = 12) and a preliminary proof of concept (POC) elution study (n = 4).

Chitosan Sponges

Chitopharm S chitosan was obtained from Chitinor AS (Tromsø, Norway) having an 82 ± 2 DDA, 251 ± 17 kDa weight-average molecular weight (MW) and 2.013 ± 0.145 polydispersity index, all manufacturer reported values. Poly(ethylene glycol) (PEG) was purchased from Sigma Aldrich (St. Louis, MO, USA) having a 6 kDa MW.

Sentrex BioSponge®, a chitosan-based approved wound dressing sponge, (Bionova Medical, Germantown, TN, USA) was included in the study due to its clinical use and commercial availability.

Chitosan sponges containing 2.0% chitosan or 1.5% chitosan and 0.5% PEG were fabricated and used in all *in vitro* studies alongside BioSponge® (Bionova Medical, Germantown, TN, USA) except for the *in vivo* implant-associated biofilm model. BioSponge was the only chitosan sponge used for the *in vivo* animal model.

Fabricated chitosan sponges, for all *in vitro* studies, were made by dissolving either 2.0% chitosan (w/v) or 0.5% poly(ethylene glycol) (PEG) and 1.5% chitosan (w/v) in 1% acidic (v/v) solutions. [188, 193] The solutions were placed in template containers with the desired shape and thickness and frozen at -20° C. Once frozen, the sponges were lyophilized, neutralized, frozen again, lyophilized again and then sterilized (Sterigenics, West Memphis, AR, USA) using low dose gamma radiation (25 – 40 kGy).

Antibiotic Dissolution

Targeted applications of the studies incorporated to test the efficacy of the Cipro/Rif combination required that the antibiotics be in solution. The dissolution of rifampin proved to be the most challenging aspect. Preliminary elution studies failed to address the solubility issue and the results (not shown) were comparable to prior studies.[172, 193] However, it was later shown that

rifampin's dissolution was not adequate for our targeted applications. Rifampin and its solubility was addressed by using 0.1 N hydrochloric acid (HCl) at 37° C with a final pH of 6.02. Scanning electron microscopy (SEM) images were taken to ensure that the solution pH value did not affect the structure or integrity of the chitosan sponges (Figs. 1, 2, and 3).

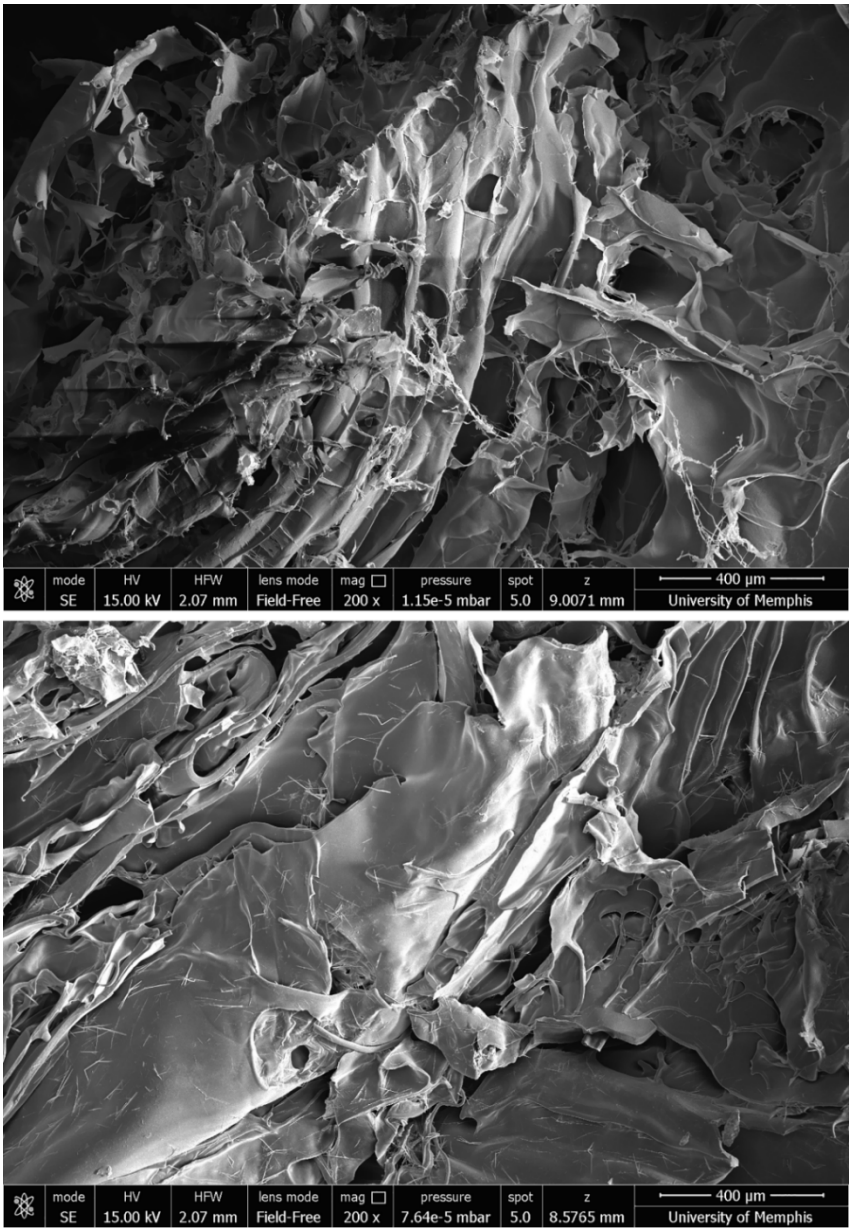

FIGURE 1.SEM images of the BioSponge pre-hydration (top) and post-hydration (bottom). The sponge was hydrated in antibiotic solution for 10 minutes, which may be comparable for times in a clinical environment. As shown the pH of the hydrating solution was not adversely breaking down the structure.

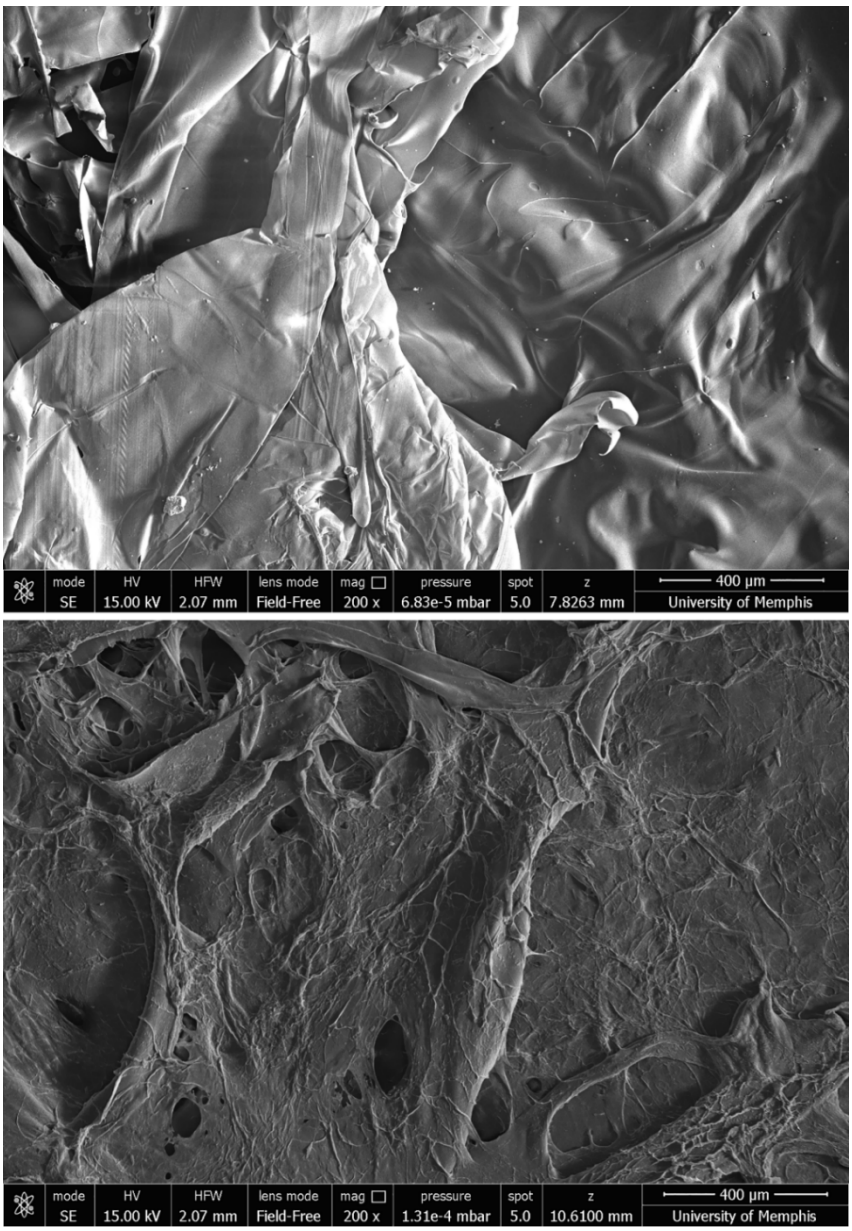

FIGURE 2. SEM images of the 0.5% Peg/1.5% chitosan sponge pre-hydration (top) and post-hydration (bottom). The sponge was hydrated in antibiotic solution for 10 minutes, which may be comparable for times in a clinical environment. As shown the pH of the hydrating solution was not adversely breaking down the structure.

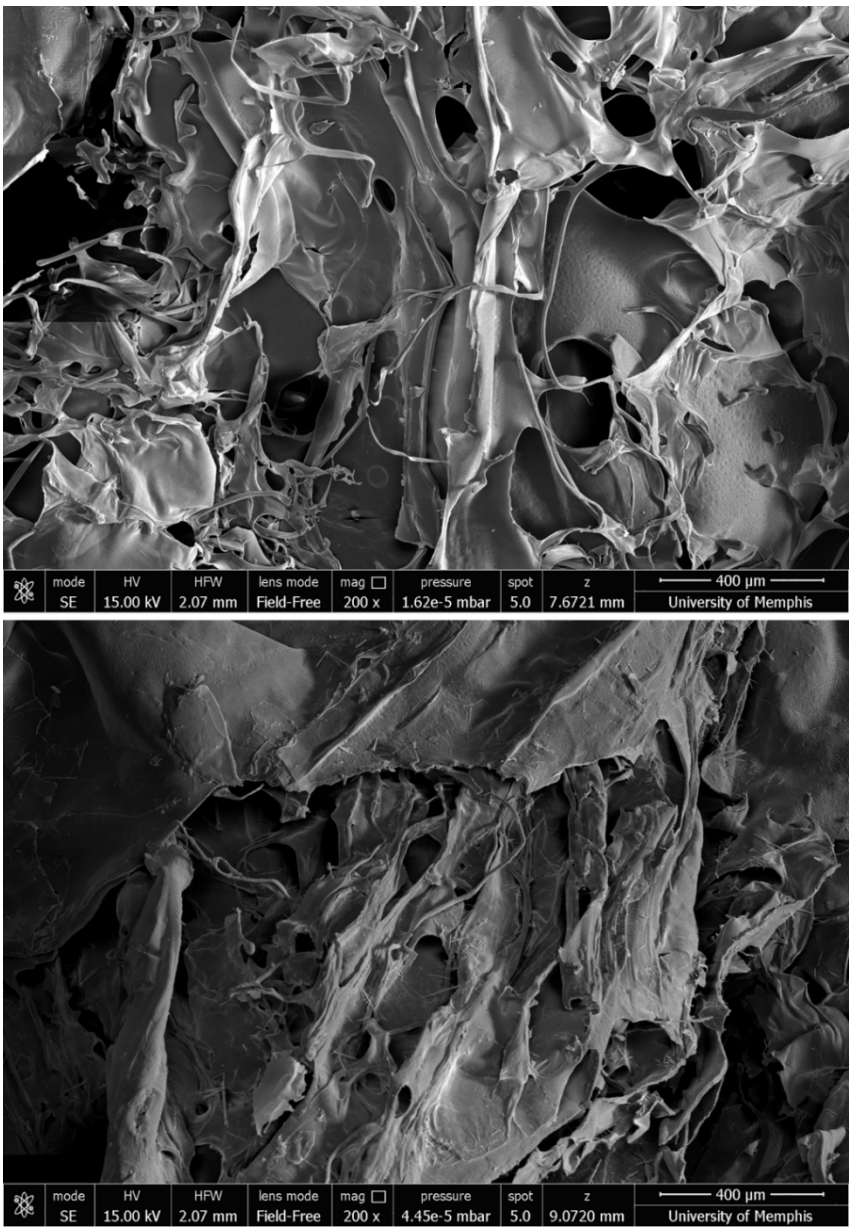

FIGURE 3. SEM images of the 2.0% chitosan sponge pre-hydration (top) and post-hydration (bottom). The sponge was hydrated in antibiotic solution for 10 minutes, which may be comparable for times in a clinical environment. As shown the pH of the hydrating solution was not adversely breaking down the structure.

In Vitro Elution

BioSponge, 2.0% chitosan, and 0.5% PEG/1.5% chitosan sponges were sectioned and weighed to nominal masses of 25 mg. The sponges were loaded with 5mg mL^{-1} of ciprofloxacin (Acros Organics, Geel, Belgium) and 5 mg mL^{-1} of rifampin (Fisher Scientific, Fair Lawn, New

Jersey, USA), the solvent was sterile 1X phosphate buffered saline (PBS). Each sponge was immersed in abundant ciprofloxacin and rifampin antibiotic solution (~10 mL) to allow hydration saturation. Sponge pre- and post-hydration masses were recorded to calculate approximate antibiotic solution volume absorbed into the samples. The sponge sections were gently placed within 125 mL NALGENE® containers with no impedance to natural structure, which allowed for adequate coverage by solution. Sterile 1X PBS (30 mL) was used to submerge the antibiotic-hydrated sectioned sponges. The submerged sponges were placed in an incubator (LabDoctor™ Mini Incubated Shaker, MidSci, St. Louis, MO, USA) at 37° C under constant motion (30 rpm). Samples of 15 mL each were taken at 1, 2, 3, 4, 5, 6, and 7 days. When the samples were taken, the PBS solution was refreshed. The samples that required PBS refreshment were returned to incubator until their time-period was reached, at which point the process repeated until all time points were elapsed. All sample collection and PBS exchange was conducted in an aseptic environment. Once samples were collected they were stored for eluate concentration analysis. Eluate sample storage was at -20 °C to prevent antibiotic degradation and maintain any active antibiotic activity.

Eluate Concentration Analysis

High-pressure liquid chromatography (HPLC), (UltiMate 3000, Thermo Scientific, West Palm Beach, FL, USA) was the analytical technique used to quantify the concentration of antibiotics in the sponge eluates. HPLC analysis for both antibiotics was modeled after a method used by Liu *et al*.[194] Chromatographic separation was achieved by using a mixture of methanol-acetonitrile-dipotassium phosphate (55 mM)-phosphoric acid (1.0M) (28:30:38:4, v/v) as the primary mobile phase (MP1), in addition to a buffer solution consisting of 55 mM K_2HPO_4 and 1.0 M H_3PO_4 (MP2). Ciprofloxacin was read at $\lambda = 280$ bandwidth of 4 with a retention time of

2.38 minutes. Rifampin was read at λ = 333 bandwidth of 4 with a retention time of 11.21 minutes. A gradient method was utilized with at a flow rate of 1.5 mL/min. Detection of ciprofloxacin occurred with MP2 at 60% and MP1 at 40% the gradient was then ramped up at 3 minutes to 100% MP1 for the detection of rifampin. The concentrations of the antibiotics were measured to determine their elution profile over time.[194-209]

In Vitro Antibiotic Activity

The antibiotic activity was completed on two separate occasions due to the solubility concern.

P. aeruginosa (ATCC 27317) was grown overnight at 37 °C in trypticase soy broth (TSB). The overnight growth was diluted 1:50 in TSB and 100 µL of this was added to plates of TSB agar and spread to make a lawn of bacteria. Once the lawn of bacteria was complete 6 mm blank discs (Beckton, Dickinson cat # 231039) were placed on the agar plates. After the placement of the disks was the addition of 20 µL of the sponge eluate to the disks. The plates were incubated for 24 h at 37 °C, pictures were taken and the diameter of the ZOI was recorded. Each plate contained five replicates for each group and time point. *S. aureus* (Cowan I ATCC 12598) was grown and tested exactly as *P. aeruginosa* except for the dilution being 1:10.

In vitro synergy assay

Checkerboard synergy testing was performed using 96-well microtiter plates. Ciprofloxacin and rifampin were dissolved in phosphate buffered saline (PBS) and diluted in tryptic soy broth (TSB) in seven 2-fold dilutions from 4 µg mL^{-1} for ciprofloxacin plated against *P. aeruginosa* (ATCC 27317) and from 2 µg mL^{-1} plated against *S. aureus* (UAMS-1). Rifampin concentrations were from 256 µg mL^{-1} against *S. aureus* (UAMS-1) and from 0.06 µg mL^{-1} against *P. aeruginosa* (ATCC 27317). Each solution was pipetted into triplicate wells of the 96-

well plate. Positive and negative controls for this experiment were inoculating TSB, without antibiotics, with and without bacteria, respectively. Each well was inoculated with *S. aureus* (UAMS-1) or *P. aeruginosa* (ATCC 27317) for a final concentration of approximately 1 x 10^4 CFUs per well. Plates were incubated for 24 hours at 37°C.

Planktonic bacteria were removed from the wells through aspiration of the liquid without disturbing the biofilm followed by gently washing with PBS three times. Heat fixation of the biofilm bacteria was achieved by heating the microtiter plate at 60°C for one hour. Once the biofilm was fixed, it was stained using 100 µL of crystal violet solution. Crystal violet not absorbed by the biofilm was removed by gently rinsing with water. At that time, a de-staining solution composed of 7.5% acetic acid, 10% methanol, and water was used to dissolve the absorbed crystal violet. Absorbance measurements were obtained at λ=540 nm using a plate reader spectrophotometer (Biotek ELx800, Winooski VT, USA). The minimum biofilm inhibitory concentration (MBIC) is the concentration of antibiotic required to inhibit the formation of biofilm.

Relationships between antibiotics was quantified using the fractional inhibitory concentration index (FICI).[210, 211] FICI was determined for each antibiotic as follows, the MBIC for the antibiotic in combination was divided by the MBIC of each antibiotic alone. The FICI for each antibiotic was summed to acquire a final FICI value. FICI values < 1 were considered synergistic, ≥ 1 and < 2 were additive, = 2 were indifferent, and > 2 were antagonistic.

In Vivo Implant-Associated Biofilm Model

All methods were approved and monitored for compliance by the animal use committee at UAMS and by the Animal Care Use and Review Office (ACURO) at the USAMRMC. A pilot

murine model with orthopaedic implant-associated infection[212] was adapted to use a polymicrobial mixture of *S. aureus* (UAMS-1)[213] and *E. coli* (ATCC 25922).[214]

In the infected mouse pin model (UAMS – IACUC 3579), sterilized chitosan sponges were hydrated with antibiotic solutions. The sponges were hydrated with 5 mL of an antibiotic solution combination of ciprofloxacin and rifampin (10 mg mL^{-1} each). NIH-Swiss mice C57BL/6, 8 - 12 weeks old, 16 – 18 grams in weight were anesthetized with Isoflurane and Avertin (400 - 600 μg g^{-1}) and the adequacy of the anesthesia was confirmed by the toe pinch reflex and the reaction to light shined into the mice eyes. The left leg was cleaned with povidine iodine and rinsed with 70% ethanol. An incision was made at the knee; a hole was drilled into the left distal femur with a 26-gauge syringe needle followed by a 23-gauge syringe needle. A sterile 1 cm x 600 μm diameter stainless steel Kirschner wire was inoculated with approximately 10^4 colony forming units (CFUs) of UAMS-1 and 10^2 CFUs of ATCC 25922 and inserted into the femur. Placement of the pin within the femur can be seen in Fig. 4.

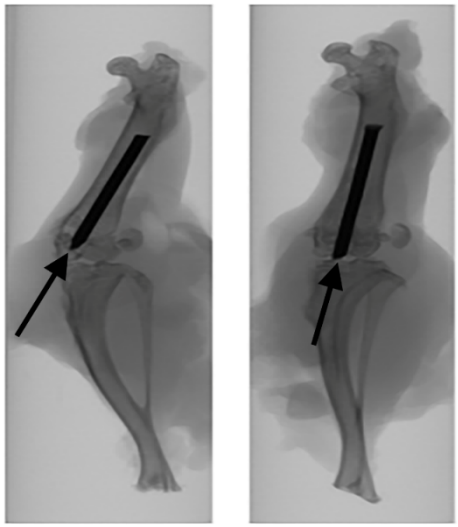

FIGURE 4. Pictured is an x-ray that shows the placement of the pin within the left femur. The arrow points to the point of insertion.

Chitosan sponges loaded with saline (negative control) or the combination of ciprofloxacin and rifampin at 10 μg mL^{-1} each were implanted adjacent to the contaminated implant (n = 12 per group). After surgical site closure, the mice were returned to their cages, monitored daily, and any that appeared moribund would be euthanized using CO_2. None of the study participants displayed any moribund attributes during the duration of the study. Animals were euthanized 7 days after the treatment, the wire implant and any associated femur tissue were removed for determination of viable bacterial CFUs remaining on/in each. The soft tissue was dissected from the bone and the femur was cut into small pieces, homogenized, and placed in a sterile saline. Wire implants were sonicated and vortexed in sterile PBS. After overnight incubation at 37° C, the homogenates were diluted, plated on agar plates, and the viable microbial colonies were counted along with the colonies remaining on the pin and bone. Bacterial clearance was defined as an apparent bacterial CFU count of zero.

Statistical Analysis

Statistical analysis of results was performed using Sigma Plot (Systat Software, Inc., San Jose, CA, USA). One way Kruskal Wallis Analysis of Variance (ANOVA) was used to determine differences between CFUs retrieved from implants, with p values of < 0.05 being considered statistically significant. Dunn's post hoc analysis was used to compare groups pairwise and determine p values. Fisher exact tests of contingency tables was used to compare the rate of clearance for implants in antibiotic-loaded groups to controls, with Bonferroni post hoc correction in cases of multiple comparisons. No other statistical analysis was performed as the principal purpose of the study was not the comparison of the various sponge types.

RESULTS

In Vitro Elution

Results from the elution study showed that rifampin elutes, virtually 100%, from its delivery device within three days (Fig. 5). Ciprofloxacin's elution profile while still displaying a burst effect was sustained throughout the duration of the study (Fig. 6).

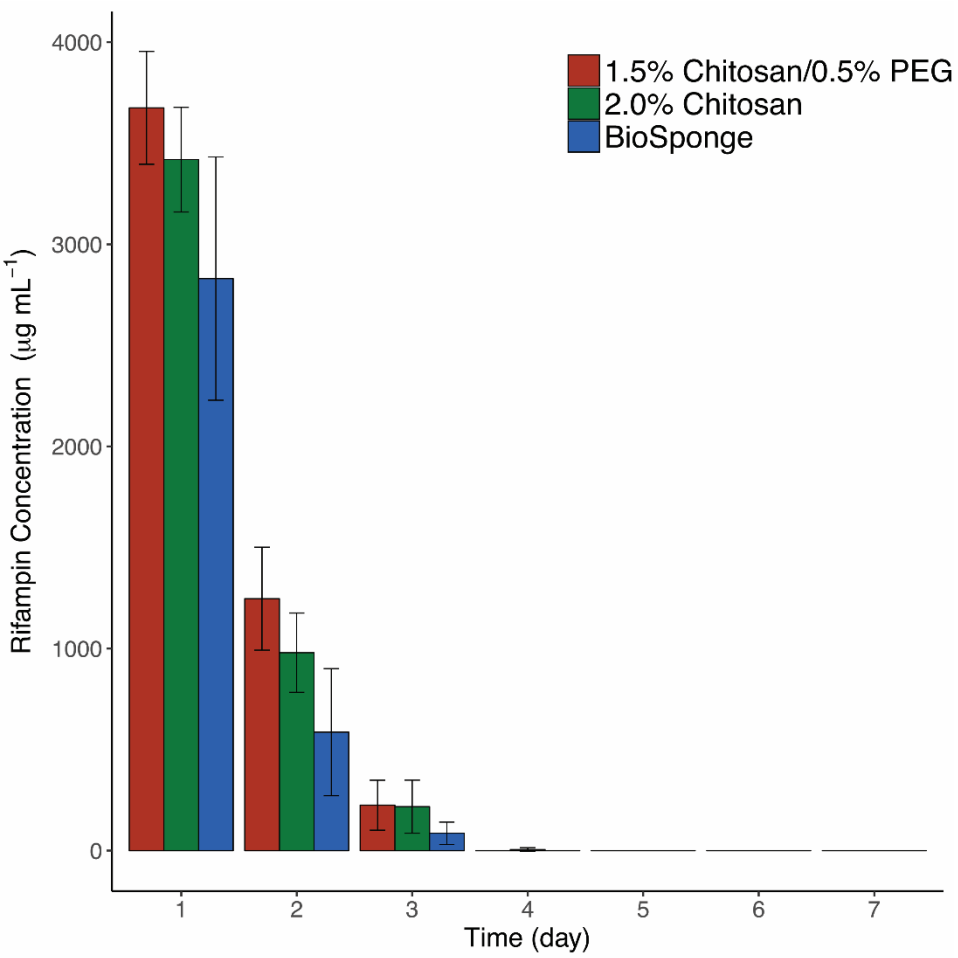

FIGURE 5. Bar graph of the mean ± standard deviation of rifampin concentration in sponge eluates (n = 5) over 7 days.

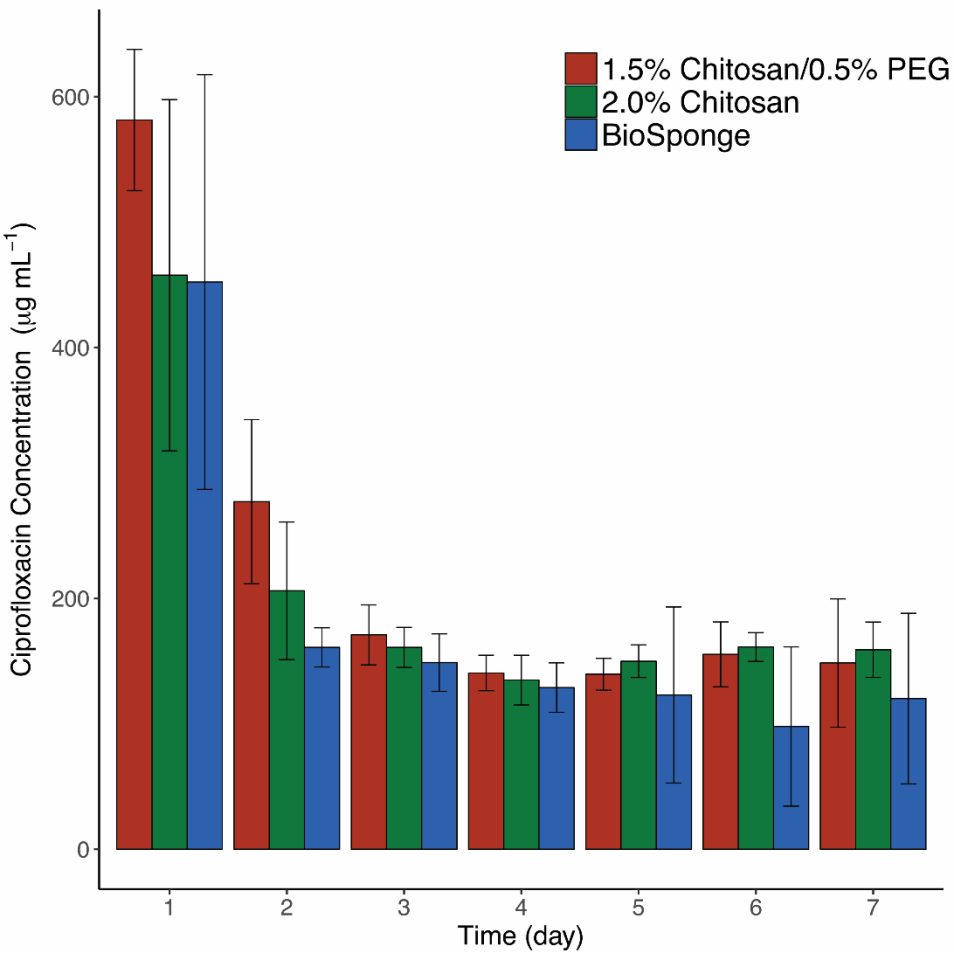

FIGURE 6. Bar graph of the mean ± standard deviation of ciprofloxacin concentration in sponge eluates (n = 5) over 7 days.

In vitro antibiotic activity

Eluates from chitosan-based local delivery devices could inhibit *P. aeruginosa* (ATCC 27317) (Fig. 7) and *S. aureus* (Cowan I ATCC 12598) (Fig. 8) through the duration of the seven-day study when solubility was addressed. Chitosan sponge eluates from proof-of-concept (POC) elution could inhibit, on average four days, *P. aeruginosa* (ATCC 27317) (Fig. 7) and *S. aureus* (Cowan I ATCC 12598) (Fig. 8).

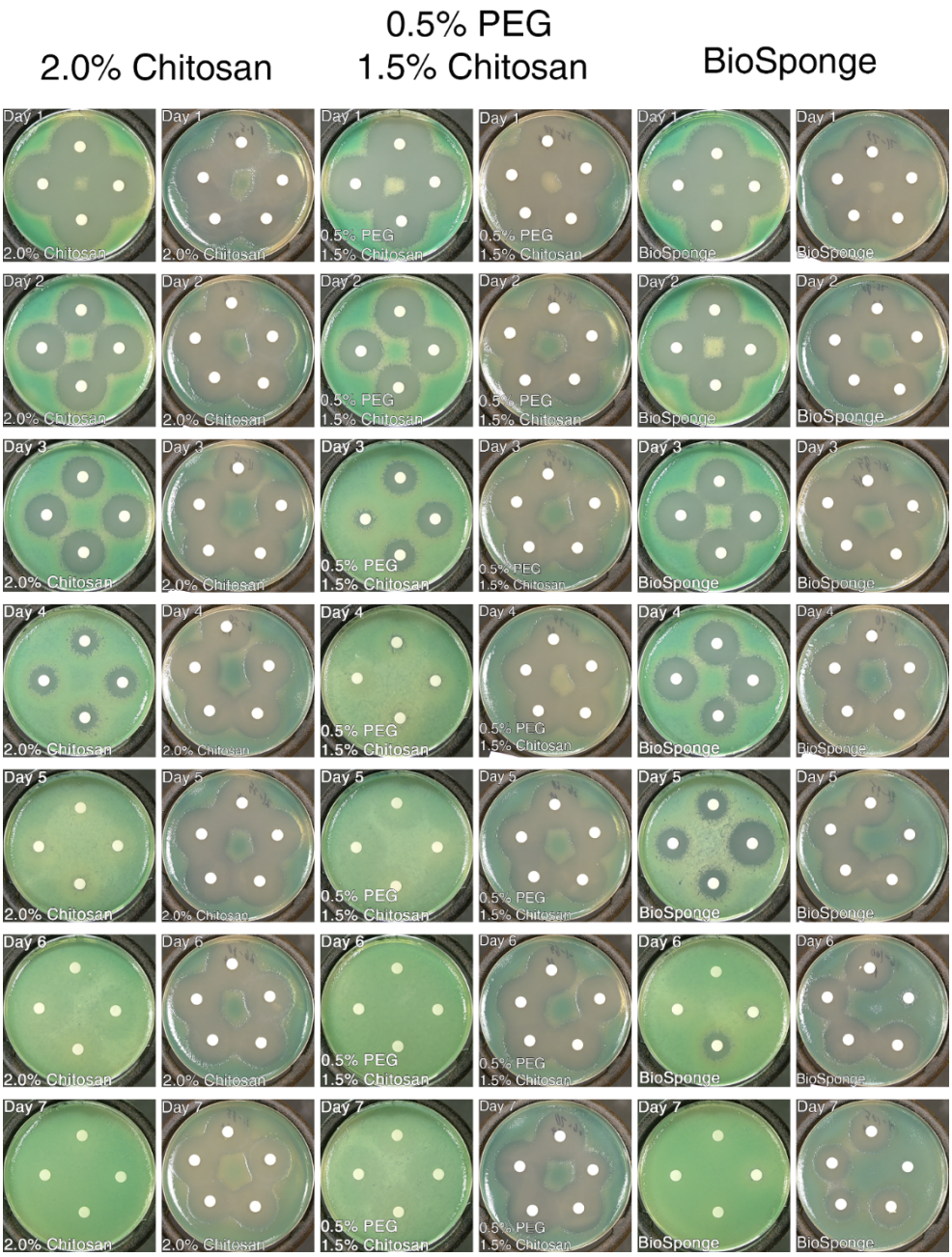

FIGURE 7. Zone of inhibition (ZOI) results of sponge eluates tested against *P. aeruginosa* (ATCC 27317). On the left of each sponge group were the results before the solubility issue was addressed. The plates on the right were achieved as the solubility of rifampin was actively addressed.

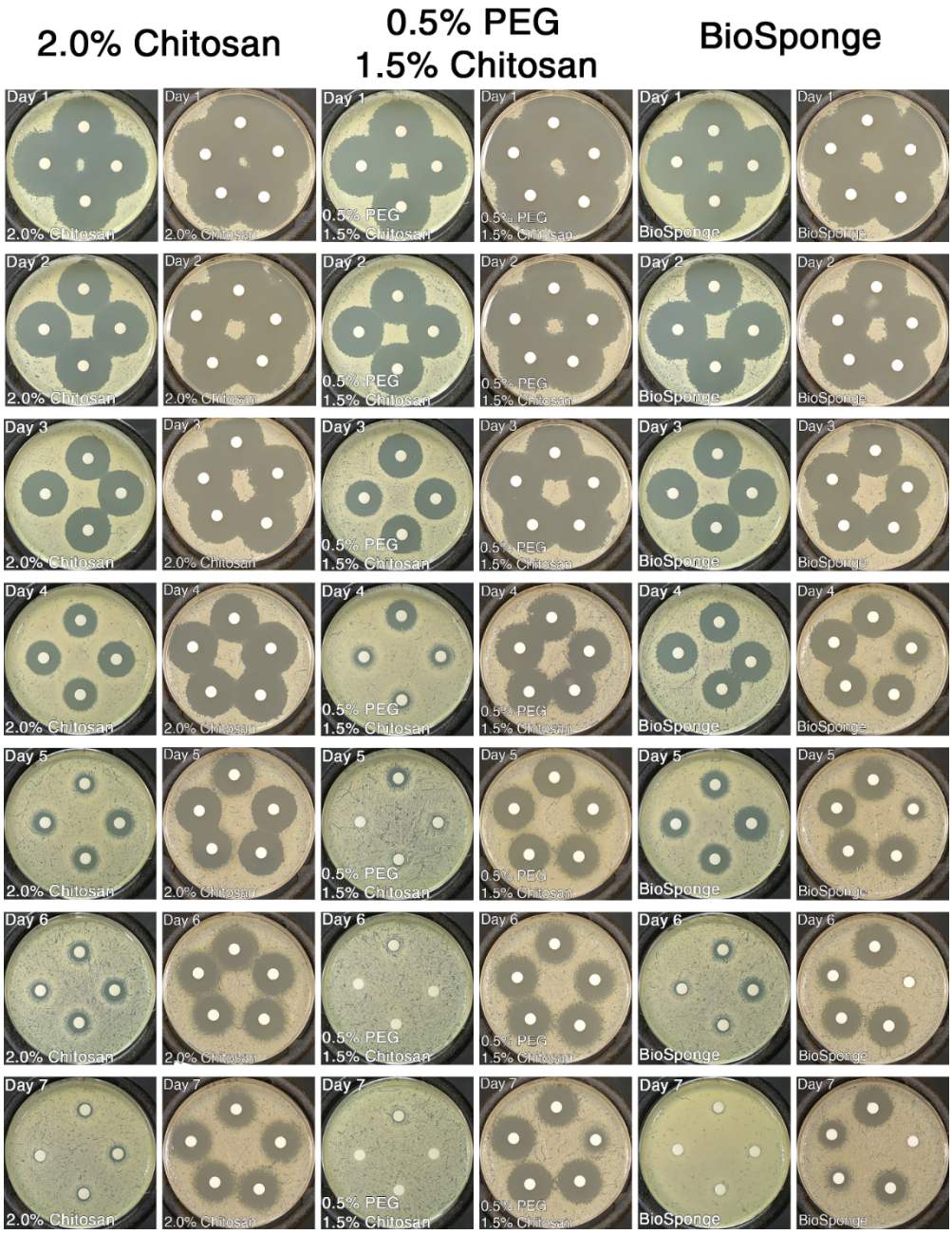

FIGURE 8. Zone of inhibition (ZOI) results of sponge eluates tested against S. aureus (Cowan I ATCC 12598). On the left of each sponge group were the results before the solubility issue was addressed. The plates on the right were achieved as the solubility of rifampin was actively addressed.

In Vitro Synergy Assay

Results from the synergy assay showed an additive effect between ciprofloxacin and rifampin against *P. aeruginosa* (ATCC 27317) with a FICI value of 1 (Table 1) and no discernible effect against *S. aureus* (UAMS-1) (Table 1).

In Vivo Implant-Associated Biofilm Model

The combination of ciprofloxacin and rifampin could completely clear both the pin (Fig. 9) and the bone (Fig. 10) of *S. aureus* CFUs at seven days. S. aureus clearance can be seen with SEM images (Fig. 11). While some *E. coli* colonies were retrieved from the bone tissue in negative saline controls without loaded antibiotics (Fig. 12), there were no viable colonies after seven days on the implant (Fig. 13).

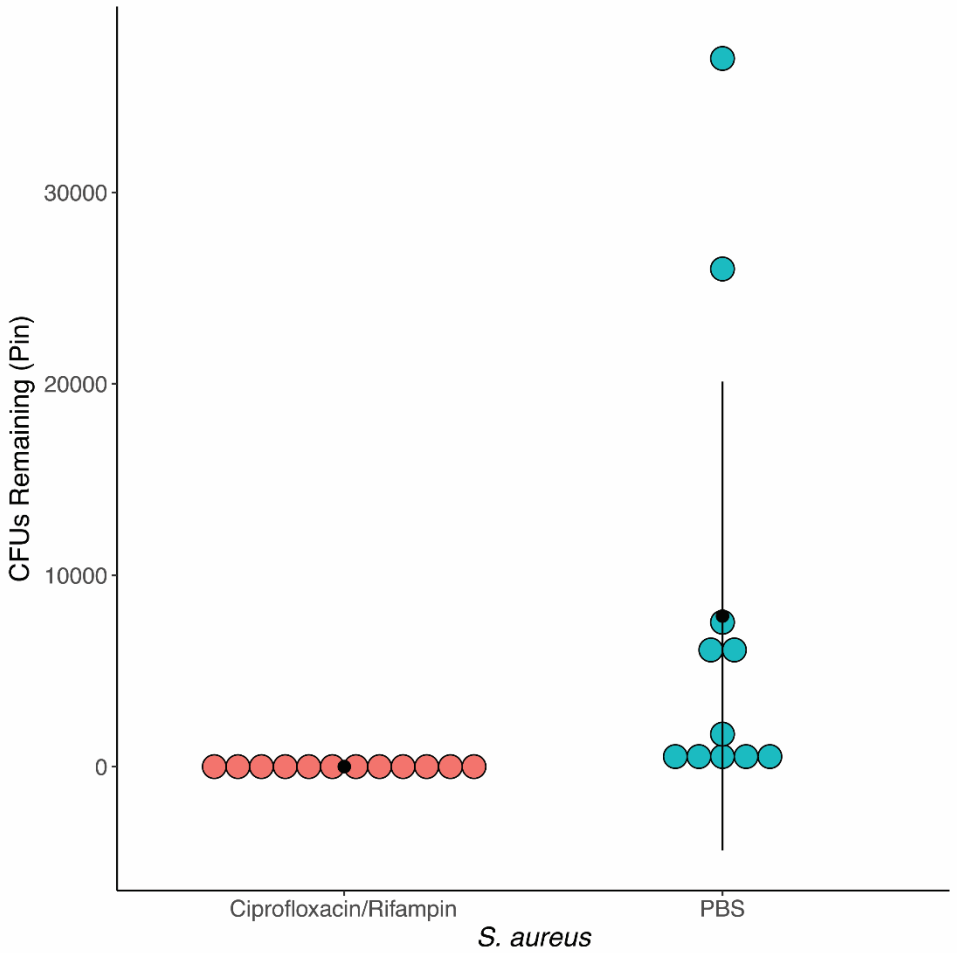

FIGURE 9. Dot plot of each animal's remaining CFUs after implant retrieval. Black dot and bar represent mean ± standard deviation for the group.

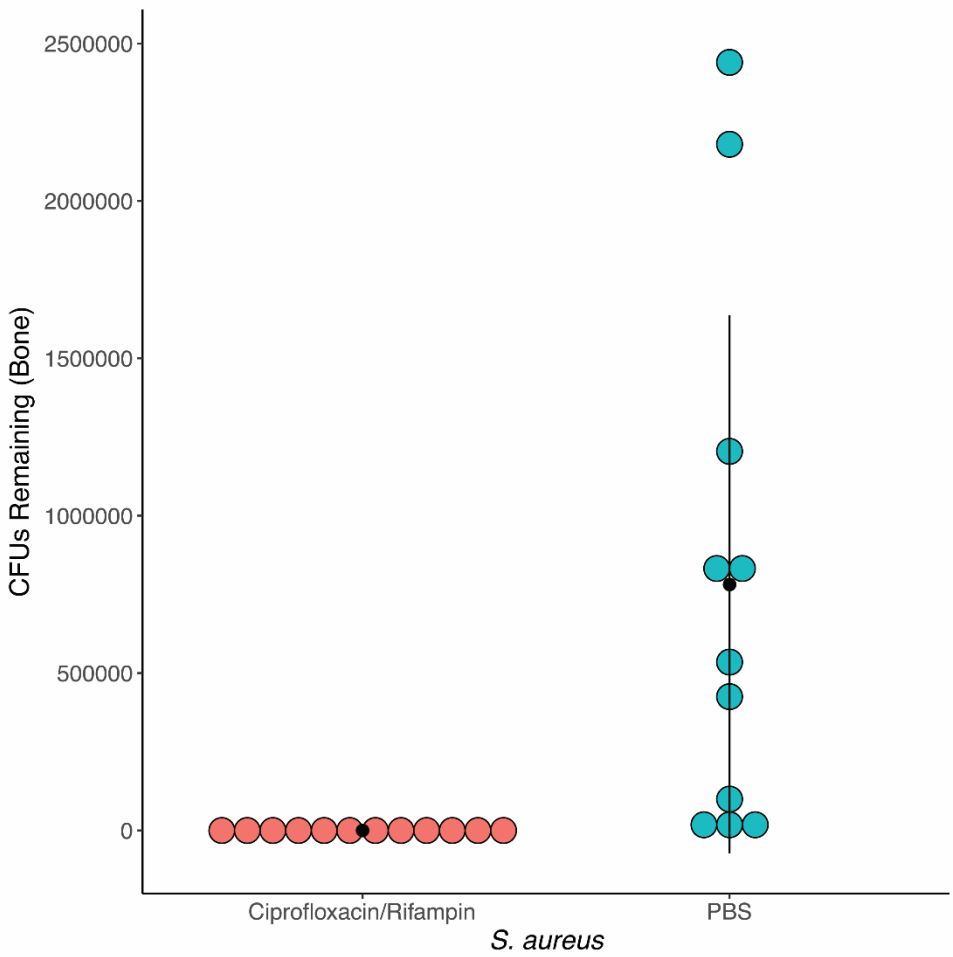

FIGURE 10. Dot plot of each animal's remaining CFUs after implant retrieval. Black dot and bar represent mean ± standard deviation for the group.

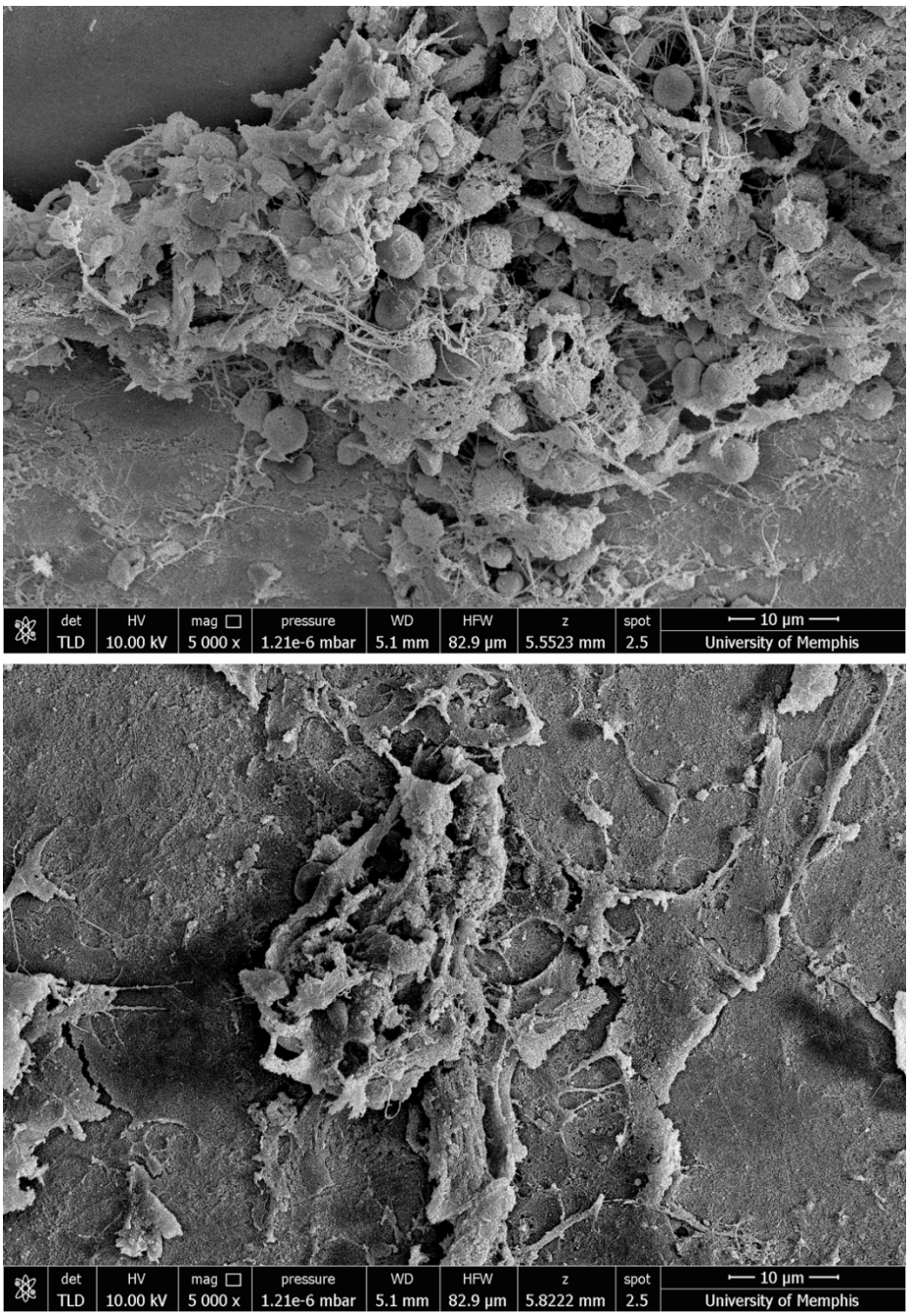

FIGURE 11. SEM image taken of the pin for a PBS pin with no antibiotic loading (top) with a discernible presence of *S. aureus*. Ciprofloxacin and rifampin treated pin (bottom).

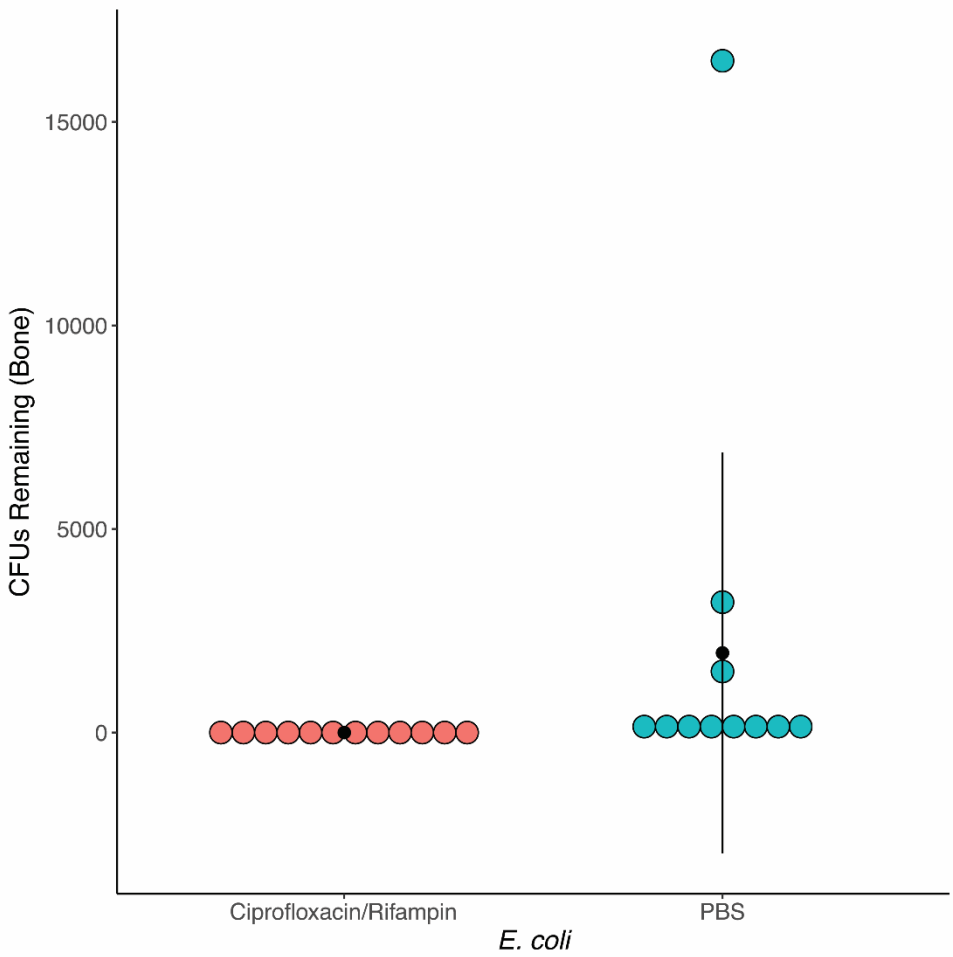

FIGURE 12. Dot plot of each animal's remaining CFUs after implant retrieval. Black dot and bar represent mean ± standard deviation for the group.

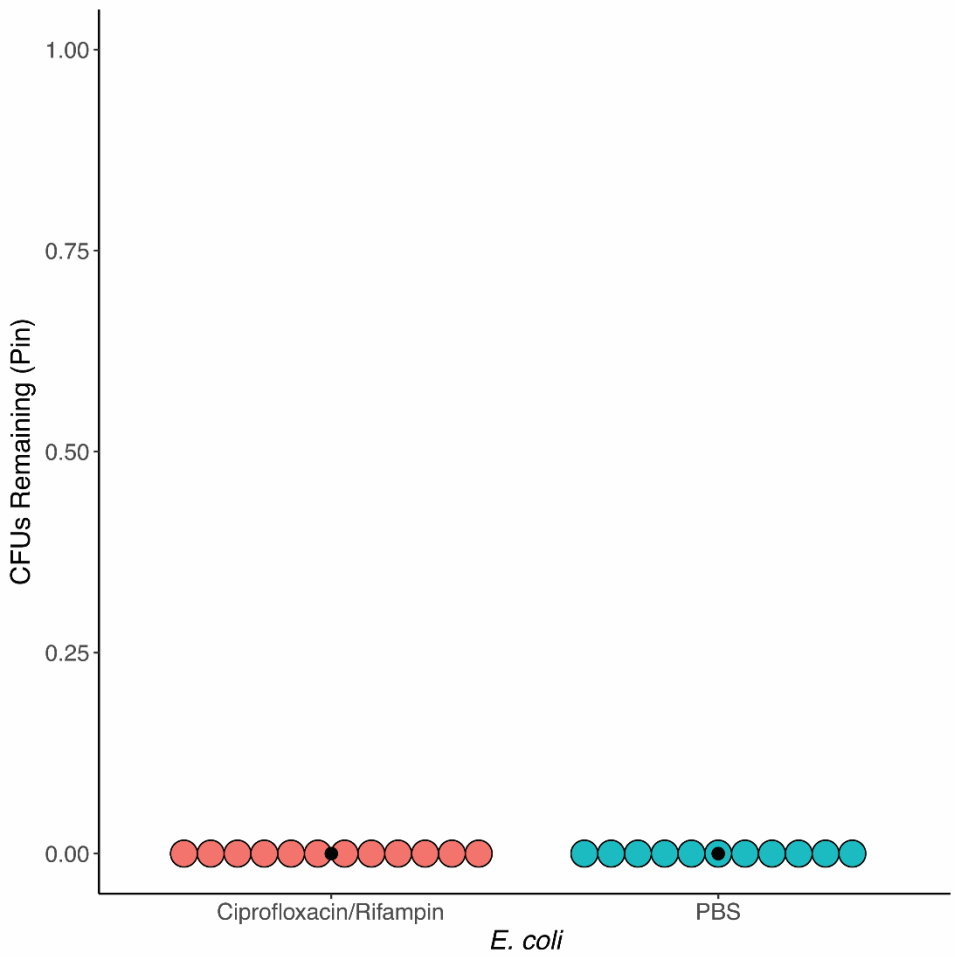

FIGURE 13. Dot plot of each animal's remaining CFUs after implant retrieval. Black dot represents mean for the group.

DISCUSSION

The primary purpose of this study was to ascertain if dual antibiotic local drug delivery from chitosan sponges could assist with inhibiting biofilm formation. During the study antibiotics were actively released from chitosan sponges at levels that were shown to be inhibitory. This study exhibits that this antibiotic combination may be introduced into a local delivery system to release active concentrations for infection inhibition. The study results also suggest a potential additive relationship against Gram-negative *P. aeruginosa* for ciprofloxacin and rifampin. Gram-negative bacteria, specifically *P. aeruginosa*, has a history of being persistent,[188, 190] treatment options consisting of antibiotics with different mechanisms of action

that are additive may eliminate the persister bacterial cells in potential clinical applications. No discernible effects, synergistic, additive, antagonistic, or indifferent, were obtained against the Gram-positive *S. aureus* due to the combination completely inhibiting the growth. While the ability to inhibit biofilm was not determined by the *in vitro* synergy assay it was shown that this combination can inhibit *S. aureus* growth. The efficacy of this treatment method was further investigated using a known model of implant infection. Results revealed complete clearance of Gram-positive *S. aureus* when dual-loaded chitosan sponges were introduced. The combination of ciprofloxacin and rifampin performance under physiological conditions, which are difficult to simulate *in vitro*, offers more validation that the combination may be a potential treatment option for practicing physicians.

The combination of ciprofloxacin and rifampin may provide improved treatment for infection prevention in musculoskeletal wounds and/or injuries. Ciprofloxacin is a 2nd generation fluoroquinolone that has been clinically effective at inhibiting some bacteria.[67-76] Quinolones/fluoroquinolones have proven to be effective against bacteria due to their ability to prevent the DNA from unwinding and duplicating.[60] Ciprofloxacin is one of the numerous fluoroquinolones that have extensive clinical usage for the treatment of Gram-negative and some Gram-positive bacterial pathogens.[63, 65, 66, 78-83] Rifampin has emerged as a major player in antibiotic combination therapy to combat biofilm among other infections. Rifamycins, represented by rifampin, specifically inhibits bacterial DNA-dependent RNA polymerase by blocking the RNA chain initiation step of bacterial DNA transcription to messenger RNA that is needed to synthesize bacterial proteins.[97,62] Rifampin is bactericidal against Gram-positive bacteria and diffuses well into biofilm where it perpetuates it bactericidal ability.[62] Disruption of biofilm is within the scope of rifampin, however rifampin-resistant mutants may be selected.[62]

Rifampin requires to be used in conjunction with other antibiotics as a result.[62] Rifampin has been tested with a number of other antibiotics for potential synergistic effects against *Acinetobacter baumannii,*[3] *Klebsiella pneumoniae*,[215] and *S. aureus*[216] among others. Ciprofloxacin has been investigated in conjunction with several antibiotics against *P. aeruginosa*[3], *Enterbacteriaceae* and *D. streptococcal*[8] to name a few. Very few studies have reported the effects of the combination of ciprofloxacin and rifampin against the strains of bacteria tested here. In a study by Zimmerli *et al*., a cure rate of 100% was achieved for those in the ciprofloxacin-rifampin group in patients with culture-proven *Staphylococcal* infection associated with stable orthopedic implants.[164] Ciprofloxacin and rifampin was the most effective treatment regimen in reduction of MRSA in bone in rats with experimental osteomyelitis.[217] The groups tested were vancomycin, vancomycin-rifampin, ciprofloxacin, ciprofloxacin-rifampin, rifampin, and a control group.[217] Widmer *et al*. concluded that combination therapy with rifampin and a quinolone should be considered for patients with orthopedic implant-related infections if the implant cannot be removed.[218] The Widmer study centered around patients with infections resulting from *staphylococci* or *streptococci*.[218]

The additive effect against *P. aeruginosa* shows promise for this combination. Complete inhibition of *S. aureus* was validated with results from most phases of the study with culmination coming from the implant-associated biofilm model. Biofilm synergy assay results for *S. aureus* were not able to be determined as the concentrations of the antibiotics completely inhibited growth. Future investigations are planned with lower concentrations of both antibiotics. There have been recent studies that were shown to completely clear *P. aeruginosa* and *S. aureus* on implants through systems consisting of phospholipid coatings impregnated with amikacin and vancomycin[219] or injectable hydrogels containing high doses of gentamicin[220], respectively. One

of the main disadvantages of chitosan sponge based local delivery systems is their dependence upon diffusion, which may result in not all aspects of an implant receiving antibiotics above the MBIC.[23, 27] Sponges have an advantage of being loaded with a customizable treatment solution immediately prior to application. Sponges do not require prefabrication in comparison with coatings or hydrogels, giving them practibility for quick interventions as needed, whether in the field or on the surgical table.[220, 221] These results extended those previously obtained that demonstrate biofilm inhibition in soft tissue implant-associated models[170] providing credence to activity in prevention of bone infection.

One major limitation for this study revolved around the *in vivo* model. This resulted from the strain of *E. coli* (ATCC 25922) used, which was not robust nor readily form biofilms. The method was adapted from an osteomyelitis model of which the investigators reported issues with the use of this specific strain.[214] In efforts to reduce or prevent severe morbidity and mortality that have previously occurred in polymicrobial models containing *S. aureus* and *P. aeruginosa*,[170] *E. coli* was selected. Lack of the robust biofilm formation greatly limits our ability to generalize conclusions to polymicrobial infections. Future *in vivo* investigations of polymicrobial biofilm will be modeled to include a more pathogenic, biofilm-forming Gram-negative bacteria, such as *P. aeruginosa* with an appropriate inoculum adaptation,[174, 222, 223] with groups receiving no local delivery of antibiotics receiving systemic delivery.

CONCLUSIONS

The ability of ciprofloxacin and rifampin, in combination, to inhibit *S. aureus* and *P. aeruginosa* growth in an *in vitro* environment and *S. aureus* and *E. coli* when released from chitosan sponges *in vivo* illustrates the clinical potential of this local delivery approach for infection prevention in extremity trauma. Complete clearance or inhibition of contaminating

biofilm pathogens may be achieved with the implementation of these antibiotics loaded within chitosan sponges.

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CHAPTER IV

DISCUSSION

Biofilm can further complicate musculoskeletal wounds normally increasing treatment time, costs, patient morbidity, and required surgeries.[1, 2, 215, 216] Antibiotic dosages that may be adverse to the patient are required to clear localized bacteria when administered systemically due to common vascular compromise in musculoskeletal wounds.[3, 8] Chitosan local delivery devices and their development were in response to a clinical need for local delivery devices there are efficacious in clearance of bacteria, biocompatible, completely biodegradable, and allow tailored point-of-care loading.[171, 188, 191, 224] Throughout the overall study these aspects were investigated to ensure that the chitosan-based local delivery systems tested actually fulfilled these requirements. Whereas, these secondary investigations were not directly related to the hypothesis however, they support a primary aspect of this research.

Multiple variations of chitosan sponges in parallel with a commercially available chitosan sponge were tested during the proof of principle phase. This was done to determine which variation would be most functional throughout the duration of the overall study. Table 1 in Appendix A displays all variations and their composition. Zone of inhibition (ZOI) sponge eluate activity results are displayed in Appendix B. Based on the results, it appears that the chitosan content affects the duration of the sponge eluate at bacterial inhibition. During this phase activity lasted on average through three days, which could be clinically relevant if all present bacteria was 100% eradicated. However, if some bacteria remain after the three days it could lead to the formation of persister cells and could result in antibiotic resistance. Parker *et al*. reported an initial burst release of loaded vancomycin from neutral chitosan/PEG and chitosan sponges after one hour, experiencing a significant decrease in eluted antibiotic thereafter.[224] An investigation by Noel *et al*. of *in vitro* release properties of vancomycin from chitosan sponges fabricated with lactic and acetic acid showed

an initial burst release of loaded vancomycin after one hour, while also experiencing a significant decrease in eluted antibiotic thereafter.[190] As demonstrated in Chapter III, sponge eluate activity could be extended throughout seven days adding credence to the use of rifampin and ciprofloxacin in a clinical application for bacterial inhibition, while Noel *et al*. found that virtually all of the loaded vancomycin was released after 72 hours.[190] Vancomycin concentrations found by Parker *et al*. were above the MIC through 24 hours, eluates utilized for turbidity testing only remained active against *S. aureus* through six hours.[224] Noel *et al*. found that the levels of 32 vancomycin eluates remained above the MIC active through 72 hours.[190] Noel *et al*. also reported amikacin eluates from chitosan sponges remaining active through 48 hours.[190] Turbidity testing performed on ciprofloxacin and rifampin loaded sponge eluates during the preliminary proof-of-principle study were active through three days. Additionally, the prior studies by Parker *et al*. and Noel *et al*. were single-loaded in comparison with the dual-loaded implemented for this study. An additional test for sponge eluate activity was conducted and its result can be seen in Appendix C. The turbidity test was conducted in parallel to the ZOI test to validate the procedure. Turbidity results confirmed what was seen with the ZOI results. Due to potential inaccurate readings, because of rifampin's color turbidity testing may not be advantageous. Rifampin, when properly in solution, will have an orange color, which could affect the absorbance, which is what is measured in turbidity.

Degradation of any local delivery device is advantageous over delivery systems that do not degrade. One of the more prevalent local delivery mechanisms practicing clinicians employ is PMMA beads, which maintain a steady stable elution profile. Some drawbacks of PMMA beads include, but are not limited to, lack of degradation and introduction of addition surface area into the surgical site or wound bed for bacterial attachment. Chitosan sponges possess the ability to biodegrade with no adverse byproducts. To confirm this two experimental degradation methods were used during the study on chitosan sponges, enzymatic and oxidative.[171] As seen in

Appendix D (Figure 19), enzymatic degradation with lysozyme was minimal whereas oxidative degradation was more robust (Figure 18) and useful in ranking degradation rates/amounts of different local delivery constructs. Oxidative testing is gaining more uses and its physiological representation is still being determined for chitosan-based systems.[171] Chitosan degradation by lysozyme has been established but to truly understand degradation of chitosan *in vivo* implantation is required and can be determined using histological methods.[171]

Chitosan sponges should be able to accept the required amounts of any tailored antimicrobial point-of-care loading necessary for desired treatment. This functional need was investigated by subjecting the chitosan sponges to a swelling ratio test. All tested sponges returned the ability to be loaded at >1000% with an aqueous solution of their initial mass, Appendix D (Table 4). Chitosan sponges possessing the ability to absorb more than 10 times their mass makes them good candidates for short-term local delivery treatment options.

Biocompatibility evaluation was conducted and reported in Chapter III but *in vitro* tests of cytocompatibility of the chitosan sponges were conducted prior and the results are seen in Appendix F. BioSponge had the highest cytocompatibility of the tested sponges at 24 and 72 hours (Figures 21 & 22). This positive outcome was expected since this product has been tested extensively by Bionova Medical and approved to be marketed as a wound dressing by the Food and Drug Administration (FDA). Study results revealed that the least cytocompatible of the tested chitosan sponges was the 2.0% chitosan sponge. There was more deviation seen in the 24-hour results compared with 72-hour. BioSponge cell proliferation exceeded the control (TCP empty well), which had no contact with any sponge. This cell number increase from this evaluation introduces the possibility that BioSponge may promote cell growth. More precise evaluations on cell function are needed for a more complete determination because cell culture does not truly duplicate the conditions and the whole story of *in vivo* compatibility. Standard deviation in the 1.5% chitosan sponges could

negate that fact that it exceeded the control at both designated times. For the 72-hour study, every sponge exceeded the control. Viable cell numbers decreased at 72-hours for BioSponge and 1.5% chitosan/0.5% PEG but increased for the 2.0% chitosan sponge in comparison to 24-hours results. This result could have come from improper loading/seeding techniques or the loading of the sponges into the wells during 24-hour study. Another possible factor could be that the cells needed time to adapt to the sponges. A longer study will be suggested for future experimentation.

Study limitations include the murine size, which has a small defect size and may not be physiologically representative of infected, complex, and large wounds in a clinical setting. Sections of the sponges were tested due to limitations in the animal wound size and in available testing parameters. Results may or may not be indicative of testing the complete sponge structure in a large complex musculoskeletal wound. However, the practicing clinician has the ability and may choose to re-size the chitosan sponge for the desired treatment plan. All *in vitro* studies were performed under controlled conditions, which may not directly correlate to *in vivo* performance. Ciprofloxacin and rifampin were tested together and not separately. This dual antibiotic use was selected and completed due to the previously reported issue that rifampin must be used in combination with another antibiotic. Most *in vitro* studies were performed during a reduced time-frame and all tested sponges during *in vitro* testing except for elution and porosity did not include the antibiotics.

CHAPTER V

CONCLUSIONS

This body of work and the studies performed within it support inclusion of ciprofloxacin and rifampin as a treatment option when loaded as a point-of-care solution. The chitosan sponge eluates showed activity through seven days which support our use as a short-term treatment option. Through local and short-term use the treatment cocktail avoids the recent released literature and FDA reports of adverse effects resulting from systemic use of ciprofloxacin. Ciprofloxacin based on results complements rifampin, which was released completely at three days in similar fashion to prior studies.

In vitro biofilm assay study results were inconclusive against *S. aureus* due to the combination completely inhibiting growth at the concentrations tested. Biofilm disruption was seen against *P. aeruginosa* offering support of the reported claim and selection of rifampin for this research. Further testing is needed at reduced concentrations, at minimum 3-fold below reported MIC values, to obtain viable results against *S. aureus*. Solubility of rifampin appeared to be temperature dependent, however contingent on specific application and treatment plan solubility may not be an issue. Recent unpublished ongoing studies are introducing powder rifampin *in vivo*, like the common vancomycin "sprinkle" and allowing physiological processes to solubilize. This proposed local delivery system required all antibiotics solubilized to load a desired concentration in the chitosan sponge. This study determined a method that allowed this to be accomplished without affecting the structure of the chitosan sponge in a detrimental way.

Rifampin and ciprofloxacin cleared all bacteria in the *in vivo* functional murine model with no adverse effects to the mice. Although the results were not without a caveat as the biofilm-forming *E. coli* strain cleared was not robust. Future testing with a more robust Gram-negative bacterium, like

biofilm-forming *P. aeruginosa*, would be required to confirm activity against polymicrobial infection.

In conclusion, a system that affords drug loading and tailored release to individual patient need and surgeon preference is advantageous in infection inhibition. The results presented in this body of work provide validation of the stated hypothesis: that engineered porous chitosan sponges, after being hydrated with ciprofloxacin and rifampin for short-term treatment, less than 14 days, would be effective at biofilm-related infection prevention. More *in vivo* testing is needed, as with most proposed treatment solutions, to confirm many of the reported conclusions, i.e. degradation, biofilm effects. With additional research the ciprofloxacin/rifampin combination may prove to be a viable option for local treatment amalgamation with standard surgical treatment of complex musculoskeletal injuries in infection prevention and biofilm-related infection prevention of surgical site infections.

CHAPTER VI

FUTURE WORK

To validate the efficacy of ciprofloxacin and rifampin when released from chitosan-based and other proven local delivery devices further *in vitro* and *in vivo* research must be conducted. Ciprofloxacin and rifampin should be investigated monogamously for elution and activity to determine if one effects the other. Solubility of rifampin with simply temperature (37° C) as a catalyst should be investigated, which may provide an easier method for complete dissolution. Investigation of pore size and structure when the chitosan sponge is singularly loaded is worth pursuing for many of the same reasons previously stated. While bacterial inhibition was the primary endpoint of the functional murine infected pin model, investigation of an established bacterial infection should be assessed during future studies. The functional infected pin model simulated metal hardware, a complement would be a functional model that simulates infected polymer implants. Although no adverse effects were seen at the antibiotic concentration used during the functional murine model future studies should address what concentrations are required for inhibition of each and proceed at those values.

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APPENDIX A: DETAILED MATERIALS AND METHODS FOR CHAPTER III

Chitosan Sponge Fabrication

Fisher Scientific (Pittsburg, PA, USA) supplied all materials unless noted. The 1.5% chitosan/0.5% PEG sponge fabrication consisted of Chitopharm S chitosan powder (Chitnor AS, Tromsø, Norway) with an 82 ± 2 DDA, 251 ± 17 kDa weight-average molecular weight (MW) and 2.013 ± 0.145 polydispersity index and polyethylene glycol (PEG) with a molecular weight of 6000 g mol^{-1} (Sigma Aldrich, St. Louis, MO, USA). The 2.0% chitosan sponge consisted of only Chitopharm S chitosan powder. Bionova Medical (Germantown, TN, USA) provided commercially available sterile BioSponge with proprietary composition. For the chitosan/PEG sponge 1% acidic acid v/v dissolved 0.5% PEG w/v in ultrapure water, additional chitosan/PEG sponges with varying chitosan content were fabricated in same manner (Table 1). The solution then dissolved 1.5% chitosan after complete dissolution of the PEG. An acid mixture of 0.75% lactic acid v/v and 0.25% acidic acid v/v dissolved 2.0% w/v chitosan in ultrapure water to make the 2.0% chitosan sponge, additional chitosan sponges with varying chitosan content were fabricated with same procedure (Table 1). Once complete dissolution was achieved 250 mL aliquots of the solutions were cast in 4 x 8 cm containers and frozen overnight at -20° C. Benchtop freeze dryers (LabConco, Kansas City, MO, USA) lyophilized the frozen solutions after they were removed from the freezer creating acidic dehydrated sponges. Sodium hydroxide (NaOH) solutions of 600 mM and 250 mM assisted in the neutralization of the acidic chitosan and chitosan/PEG sponges, respectively. Sponges were covered in the appropriate NaOH solution for approximately seven minutes. At the expiration of the seven minutes the NaOH solution was removed and the sponges were washed with ultrapure water until their eluate tested for a neutral pH. Neutral sponges were then frozen again at -20° C for one hour and lyophilized

to create the neutral unsterilized sponges. Sterigenics (West Memphis, AR, USA) sterilized all fabricated twice lyophilized neutral using low dose gamma radiation (25 – 40 kGy).

Table 1. Composition of various chitosan sponges used during proof of principle phase of study.

Chitosan Content	*Peg Content*	*Acid Content*	*Document Reference*
1%	N/A	1% (0.75% LA, 0.25% AA)	1C
1.5%	N/A	1% (0.75% LA, 0.25% AA)	1.5C
2%	N/A	1% (0.75% LA, 0.25% AA)	2C
0.5%	0.5%	1% AA	PC
1.0%	0.5%	1% AA	1PC
1.5%	0.5%	1% AA	1.5PC
BioSponge (proprietary content)			B

***Poly(ethylene glycol) (PEG), Acetic Acid (AA), Lactic Acid (LA)**

Antibiotic Dissolution

Rifampin (Fisher Scientific, Fair Lawn, New Jersey, USA) and ciprofloxacin·HCl (Acros Organics, Geel, Belgium) were weighed out, independently, to make 5 mg mL^{-1} solutions in ultrapure water. A 0.1 N HCl solution (25° C) was prepared in ultrapure water and 100 mL was added to a beaker. The temperature was raised to 37° C, rifampin, and 100 mL of ultrapure was added, temperature was brought to 37° C. If any rifampin was not completely in solution drops of 0.1 N HCl was added at 37° C until complete dissolution was achieved. Once complete dissolution of rifampin occurred, the measured amount of ciprofloxacin was added to the solution and the pH was recorded. A solution of 500 mM NaOH (25° C) in ultrapure water was prepared. Drops of the NaOH were added to the solution to increase the pH to an acceptable range that would not affect the chitosan sponge structure. The final pH of the antibiotic solution at 37° C was 5.86. Attempts to increase the pH above 5.86 resulted in antibiotics falling out of solution. The solution was transferred to an appropriate volumetric flask and ultrapure water was added to complete the solution.

In vitro elution

All procedures contained within this section were conducted within an aseptic environment. All sterilized sponges were sectioned to and weighed to nominal masses of 25 mg. Dehydrated sectioned sponges (n = 5) were separately placed in containers with ~10 mL of the 5 mg mL^{-1} ciprofloxacin/rifampin antibiotic solution for 10 minutes. Sponges were carefully removed from the container and gently placed in 125 mL NALGENE containers, which had adequate space for the sponges to keep their natural structure. The mass of the newly antibiotic hydrated sponges was recorded.

Sterile 1X phosphate buffered saline (PBS) was made from 10X PBS, with ultrapure water and a Sterilmatic autoclave (Market Forge, Boston, MA, USA). Sterile 1X PBS (30 mL) was placed in each container, which fully submerged the sponge. Containers were then placed in an incubator (LabDoctor Mini Incubated Shaker, MidSci, St. Louis, MO, USA) at 37° C under constant motion of 30 rpm. At predetermined time intervals of 1 – 7 days, systems were removed daily from the incubator and samples of 10 mL, in 2 mL aliquots, were taken. Systems that were slated for the day of sample were discarded. Systems that were not slated for the day of sample, had an aspirator remove the excess fluid, PBS was restored, and the systems were placed back into the incubator at the same conditions as previously stated. This process continued until day seven at which point all systems had been properly sampled and discarded. Eluates were stored immediately after collection in a freezer at -20° C until further analysis.

Eluate Concentration Analysis

High-performance liquid chromatography (UltiMate 3000, Thermo Scientific, West Palm Beach, FL, USA), formerly known as high-pressure liquid chromatography, determined the concentration of ciprofloxacin and rifampin in the sponge eluates. Stock concentrations of 1 mg

mL^{-1} were made for ciprofloxacin and rifampin for the standard curve and to randomly verify detection method throughout the sequence. HPLC grade methanol (30%), HPLC grade acetonitrile (28%), 55 mM dipotassium phosphate (38%), and 1.0 M phosphoric acid were combined to create the primary mobile phase (MP1). The primary mobile phase implemented to detect both ciprofloxacin and rifampin was modeled after Liu *et al.*[194] Detection of both antibiotics with one primary mobile phase required an additional buffer solution (MP2) which consisted of 1.0 M phosphoric acid and 55 mM dipotassium phosphate. The UltiMate 3000 has four pumps which made this adaptation possible. MP1 was placed on one pump and MP2 was fed through another pump.

To ensure MP1 accurately detected targeted antibiotics 1.5 mL of stock concentrations were placed in 2 mL HPLC vials and analyzed. Now only MP1 was used to detect the targeted antibiotic ciprofloxacin or rifampin. Once UV-Vis detection, using 2 μL injections, of each (n = 10) was validated by same retention time, height, and sharpness of peak, a gradient method needed to be optimized to detect both analytes in each sample with one pass. This is necessary if the retention time of the antibiotics are close enough to introduce potential interference, possibly skewing results. The adapted MP1's primary use was detection of rifampin therefore the focus was on ciprofloxacin. Optimization required adjusting the amount of MP1 and MP2 to percentages that efficaciously detected ciprofloxacin. After numerous iterations, the final percentage that detected ciprofloxacin was 60% MP2 and 40% MP1. The new detection parameter required validation for detection of both ciprofloxacin and rifampin. A new stock concentration (1.5 mL) that consisted of 1 mg mL^{-1} ciprofloxacin and 1 mg mL^{-1} rifampin was placed into 2 mL HPLC vial for analysis. For efficacious analysis of sponge eluates, a gradient method needed optimization. To accomplish this all the previously mentioned stock

concentrations were implemented. The chromatographic separation detection gradient method required numerous iterations before determining one that returned the desired detection parameters. The gradient method determined for this analysis consisted of a four-minute equilibration to ensure 40% PM1 and 60% PM2 at which point the injection was initiated. This was held steady through three minutes at which point the gradient was ramped up to 100% PM1 and held there for the duration (12 minutes) of the method. Validation of said gradient method required detection of ciprofloxacin and rifampin from either of the vials in a succession of 25. The flow rate was 1.5 mL min^{-1} with a BDS HYPERSIL C18 reverse phase column (Thermo Scientific, West Palm Beach, FL, USA), held at 30° C set by the method and possible due to the UltiMate 3000. Ciprofloxacin was read at $\lambda = 280$ with a bandwidth of 4 and a retention time of 2.38 minutes. Rifampin was read at $\lambda = 333$ with a bandwidth of 4 and a retention time of 11.21 minutes. Sponge eluate samples were thawed and were plated in a 96-well plate (250 µL). Each 96-well plate contained two columns of known concentrations for continued method validation throughout the sequence. Preliminary proof of principle results are displayed in Figures 14 & 15.

Preliminary Results

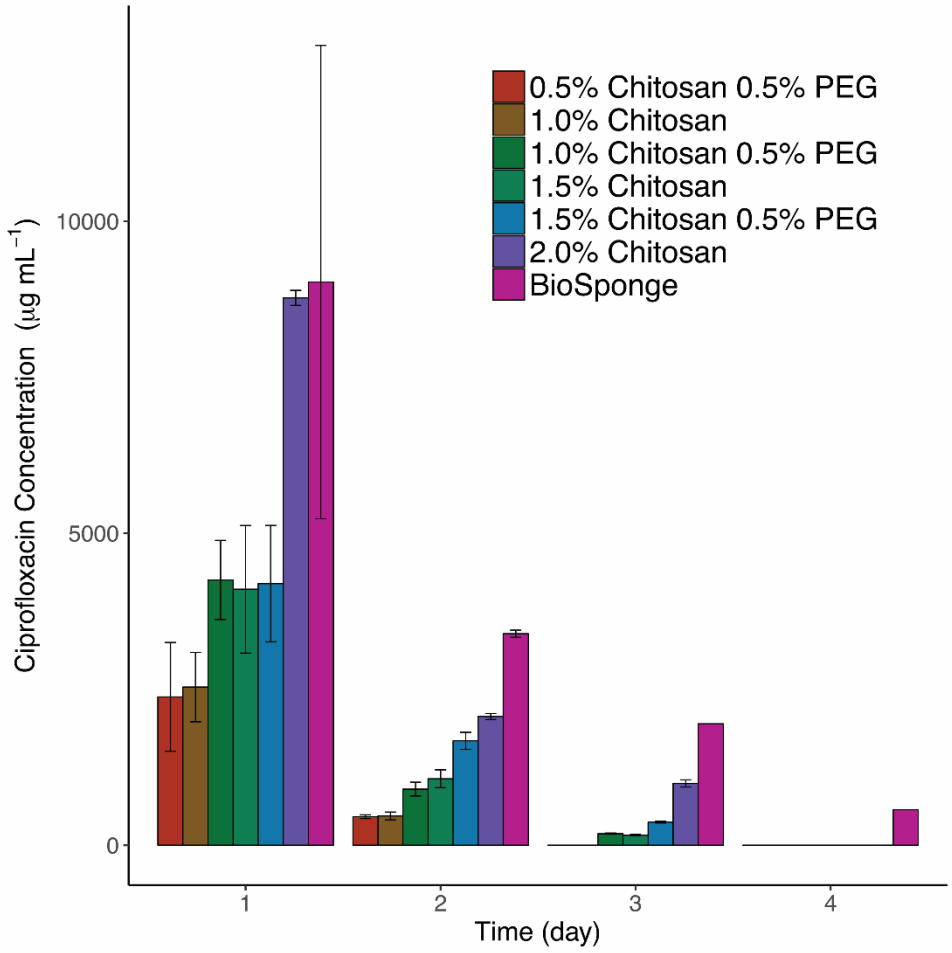

FIGURE 14. Bar graph of mean ± standard deviation of ciprofloxacin concentration in sponge eluates (n = 4) during preliminary testing.

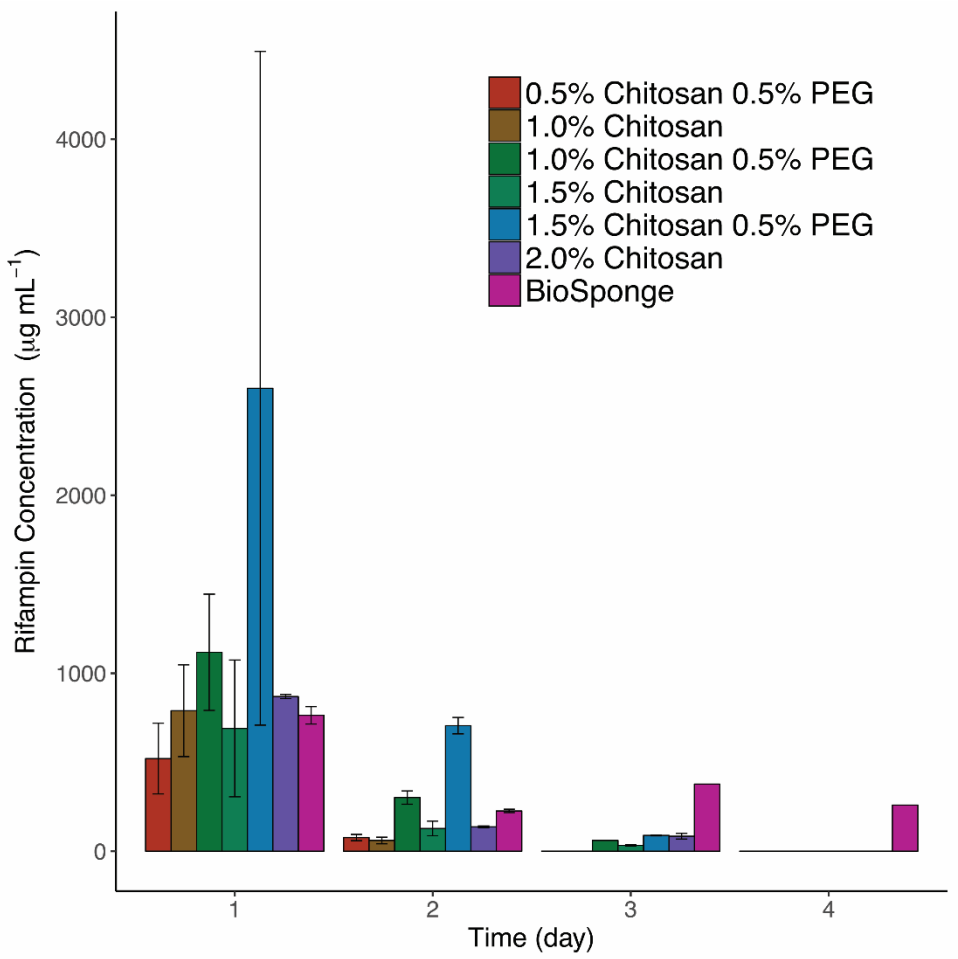

FIGURE 15. Bar graph of mean ± standard deviation of rifampin concentration in sponge eluates (n = 4) during preliminary testing.

Scanning Electron Microscope Image Acquisition (Sponge Pore Structure)

The dehydrated sponges (n = 4) were sectioned to 6.25 cm^2 with half remaining dehydrated and others loaded with the ciprofloxacin/rifampin antibiotic cocktail (pH 5.86). Samples were loaded independently for 10 minutes with excess solution removed by aspiration. Sponge samples needed to be further reduced in size for imaging purposes using a Nova Nano scanning electron microscope (SEM) 650 Field Emission System. Liquid nitrogen was implemented to flash freeze the samples allowing for smaller pieces to be cracked from them with tweezers. The samples were placed in a dewar and placed in liquid nitrogen until frozen.

Pore structure inside the sponge, which would have been harmed by cutting, was preserved using this method. Once the samples were of appropriate size, they were attached to SEM sample holders using double-sided carbon tape and dried in a vacuum oven (21 inHg) for 72 hours at 40° C. Immediately prior to imaging, samples were coated with a 10 nm layer thick of gold-palladium. Surface area and cross sectional images were taken with an excitation voltage of 15.00 kilovolts at magnifications of 200x.

APPENDIX B: ZONE OF INHIBTION PHOTOGRAPHS

Pseudomonas aeruginosa

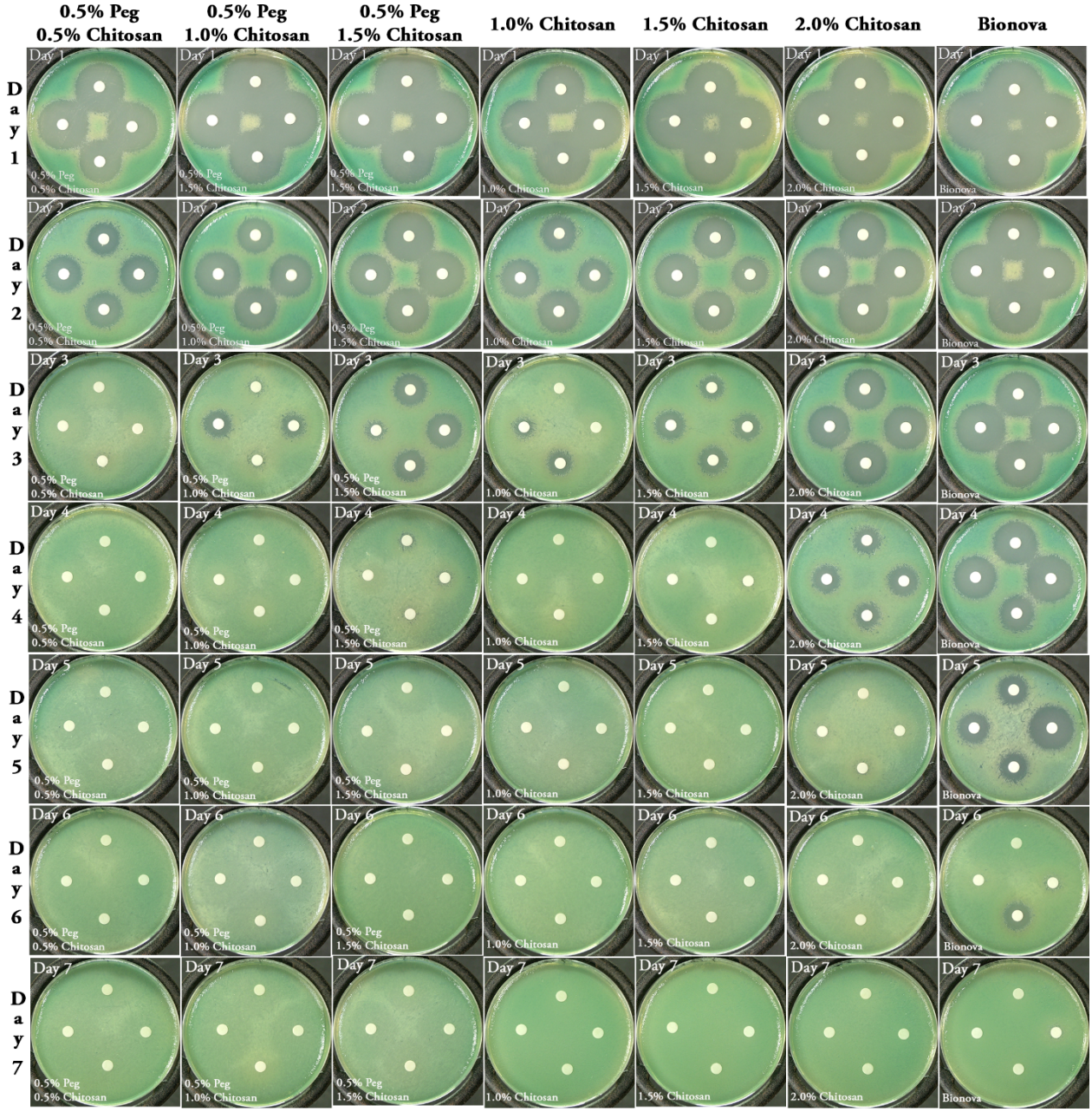

FIGURE 16. Zone of inhibition photographs from the initial preliminary proof of principle elution study testing against *P. aeruginosa*.

Staphylococcus aureus

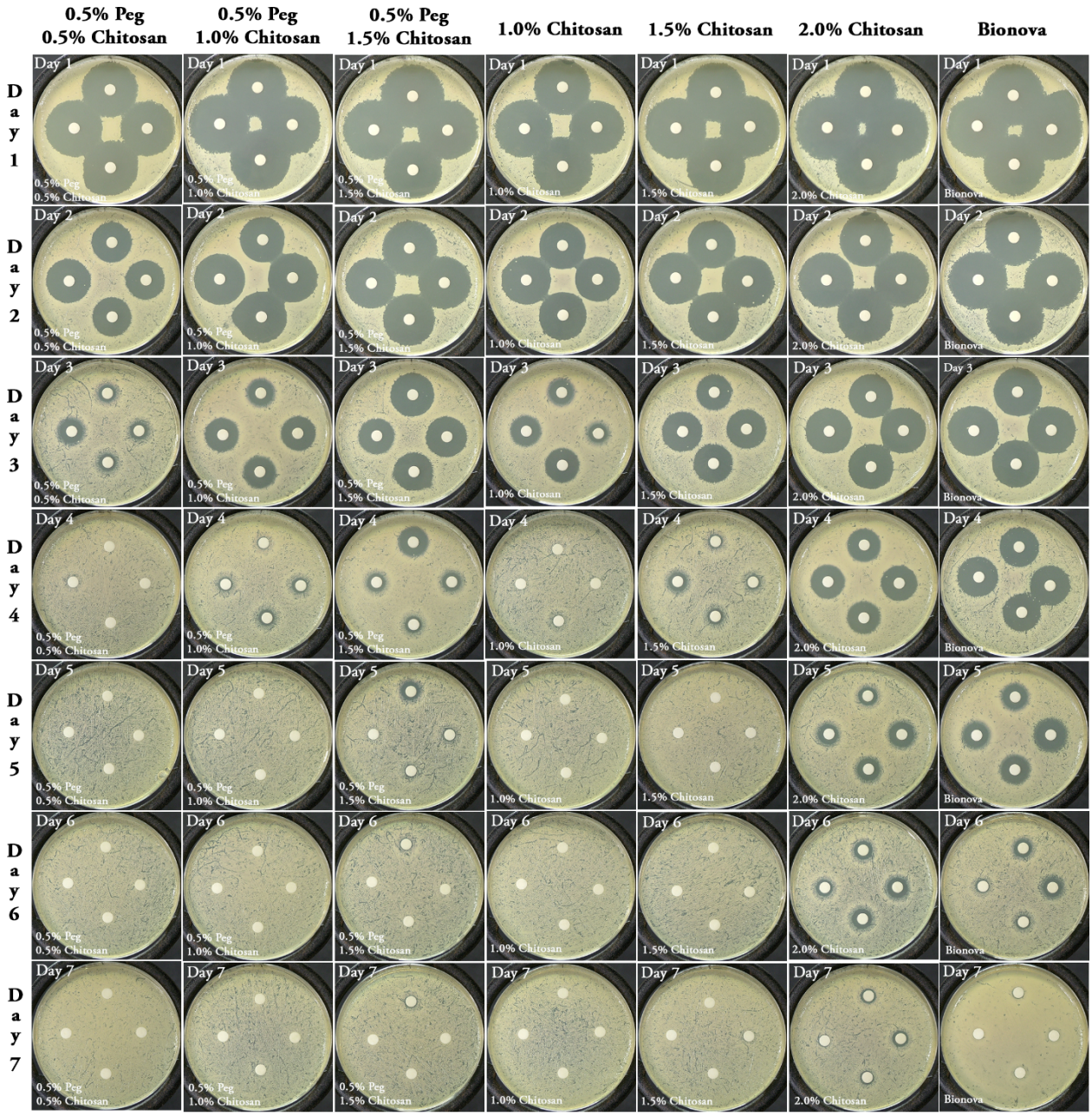

FIGURE 17. Zone of inhibition photographs from the initial preliminary proof of principle elution study testing against *S. aureus*.

APPENDIX C: CHITOSAN SPONGE ELUATE TURBIDITY

Rationale

Bacteria reside *in vivo* within an aqueous environment. Blood and other aqueous solutions provide transport possibilities of bacteria from one location to another.[170, 188, 193] Turbidity testing is a pseudo model of this clinical scenario. Eluate samples are tested against bacteria floating in an aqueous environment to determine whether the treatment can inhibit growth within solution.

Pseudomonas aeruginosa Turbidity

P. aeruginosa (Schroeter) Migula ATCC 47085, commonly referred to as PAO-1, was the bacterial strain used during this testing process. The bacterium was diluted 1:50 in TSB and grown overnight in an incubator at 37 °C. The eluate sample (200 µL) was placed into a 5 mL polystyrene round-bottom tube (Falcon) with 1.75 mL TSB, and 50 µL PAO-1. Positive controls consisted of 1.95 mL TSB and 50 µL PAO-1, while negative controls contained 1.8 mL TSB and 200 µL eluate sample. The tubes containing the mixtures was placed in an incubator at 37 °C for 24 h. Once the 24 h were passed the tubes were removed from the incubator and absorbance was read at $\lambda = 540$ on a Spectronic 200 (Thermo Scientific, Waltham, MA, USA). The results can be seen in Table 2.

Staphylococcus aureus Turbidity

S. aureus ATCC 49230 (osteomyelitis clinical isolate), often referred to as UAMS-1, was the bacterial strain used during this testing procedure due to its confirmed contribution to bone infections, being cultured from osteomyelitis. The bacterium was diluted 1:5 in TSB and grown overnight in an incubator at 37 °C. The eluate sample (200 µL) was placed into a 5 mL polystyrene round-bottom tube (Falcon) with 1.75 mL TSB, and 50 µL UAMS-1. Positive

control consisted of 1.95 mL TSB and 50 µL UAMS-1, while negative controls contained 1.8 mL TSB and 200 µL eluate sample. The tubes containing the mixtures were placed in an incubator at 37 °C for 24 h. After the 24 h had passed the tubes were removed from the incubator and absorbance was read at λ = 540 nm on a Spectronic 200. The results can be viewed in Table 3.

Results

The results were in agreeance with the ZOI results seen in Appendix B. During this phase of the investigation no efforts were made to address the solubility of either antibiotic. The results were still comparable to other previous tested antibiotic combinations and their eluates from chitosan sponges.

Table 2. Results of turbidity testing for *Pseudomonas aeruginosa*, (-) indicates inhibition and (+) indicates growth.

Day	0.5% Peg 1.5% Chitosan	2.0% Chitosan	BioSponge
1	-	-	-
2	-	-	-
3	-	-	-
4	+	+	-
5	+	+	-
6	+	+	+
7	+	+	+

Table 3. Results of turbidity testing for *Staphylococcus aureus*, (-) indicates inhibition and (+) indicates growth.

Day	0.5% Peg 1.5% Chitosan	2.0% Chitosan	BioSponge
1	-	-	-
2	-	-	-
3	-	-	-
4	+	-	-
5	+	+	+
6	+	+	+
7	+	+	+

APPENDIX D: CHITOSAN SPONGE DEGRADATION

Rationale

Degradability of chitosan sponges is an proposed advantage in clinical applications.[171, 172, 225-227] For the purpose of this study, degradation timing shortly after functional elution completion would be optimal. Degradability was ascertained using two methods, enzymatic and oxidative.[135, 172, 188, 226, 228-234] For both degradation studies sponges were be fabricated and sectioned as they were for the elution study and SEM imaging (APPENDIX A). Oxidative accelerated *in vitro* degradation aids in the determination of overall degradation of the sponge types for further characterization of longer term *in vivo* degradation in reduced time frames and was investigated as a result.[235-238] *In vitro* enzymatic degradation testing with lysozyme has been established as a physiological representation of *in vivo* degradation.[135, 239-242]

Oxidative Degradation

Sponges used for this phase of testing were fabricated or obtained as detailed in Appendix A. Sectioned sponge masses were recorded. Sponges were hydrated and had *in vitro* oxidative degradation evaluated in bacteriological petri dishes 100 x 15 mm (Falcon, Corning, NY, USA). The samples were hydrated for 10 minutes in 20 mL sterile 1X PBS; excess solution was removed by aspiration. Once the sponges were hydrated they were submerged in 30 mL of solution consisting of 1x sterile PBS, 3% H_2O_2, and 0.1 M $CoCl_2$. Upon submersion, samples were placed at 37 °C in the LabDoctor Mini. Samples were collected at 3.6, 7.2, 10.8, 14.4 and 18 hours from the beginning of the study. Since this degradation method is accelerated the time points (18 hours ÷ 5) were adjusted to mimic (ten days sampled every two days) those needed for enzymatic degradation with lysozyme. Solution was refreshed at all collection intervals. Collected samples were placed in an oven at 80 °C and allowed to thoroughly dry. When dried,

the mass of the sponges was measured and recorded. Oxidative sponge mass percent remaining can be viewed in Figure 18.

Enzymatic Degradation

Sponges used for this phase of testing were fabricated or obtained as detailed in Appendix A. Sample sponges (n = 4) were sectioned to 9 cm^2. Sectioned sponge masses were recorded. Sponges were hydrated and had *in vitro* enzymatic degradation evaluated in Falcon bacteriological petri dishes 100 x 15 mm. The samples were hydrated for 10 minutes in 20 mL sterile 1x PBS; excess solution was removed by aspiration. Once the sponges were hydrated they were submerged in a 30 mL of solution consisting of 1x sterile PBS, 1 mg mL^{-1} lysozyme, and 1% antibiotic/antimycotic (AB/AM), containing penicillin, streptomycin, and amphotericin B. After submersion, the sponges were placed in the LabDoctor Mini at 37° C. The samples were collected every two days for duration of study (10 days). When samples were collected all PBS/lysozyme solution was refreshed in uncollected samples. Uncollected samples were returned to incubator until the next sample collection time was attained, at which time the process repeated until all predetermined time points were achieved. Collected samples were placed in an oven at 80° C and allowed to completely dry. Once dried, the mass of the sponges was measured and recorded. Enzymatic sponge mass percent remaining can be viewed in Figure 19.

Results

The degradation was linear for the oxidative through four time points. Clear degradation advantages were seen for the chitosan sponge that contained PEG, however the purpose was not to compare sponge type it was to show biodegradable properties relating to chitosan sponges.

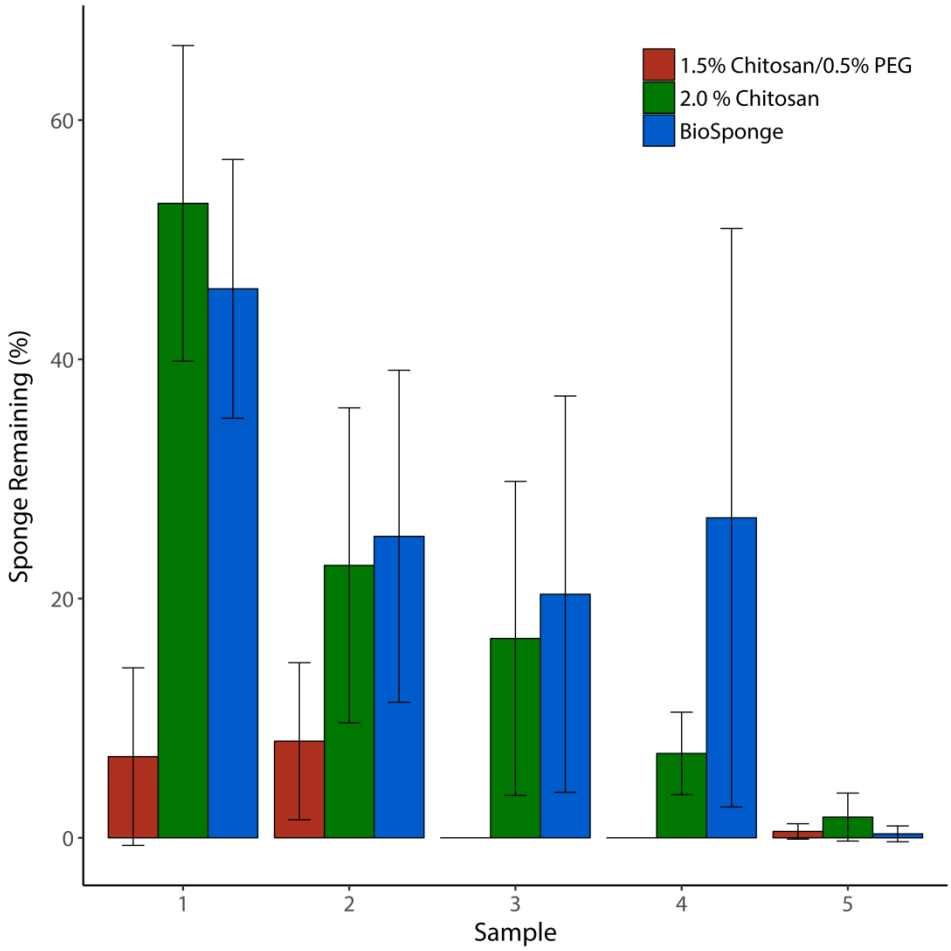

FIGURE 18. Bar graph of mean ± standard deviation of sponge remaining mass as a percentage when subjected to oxidative mediated testing. Five samples were taken over the course of 18 hours, every 3.6 hours.

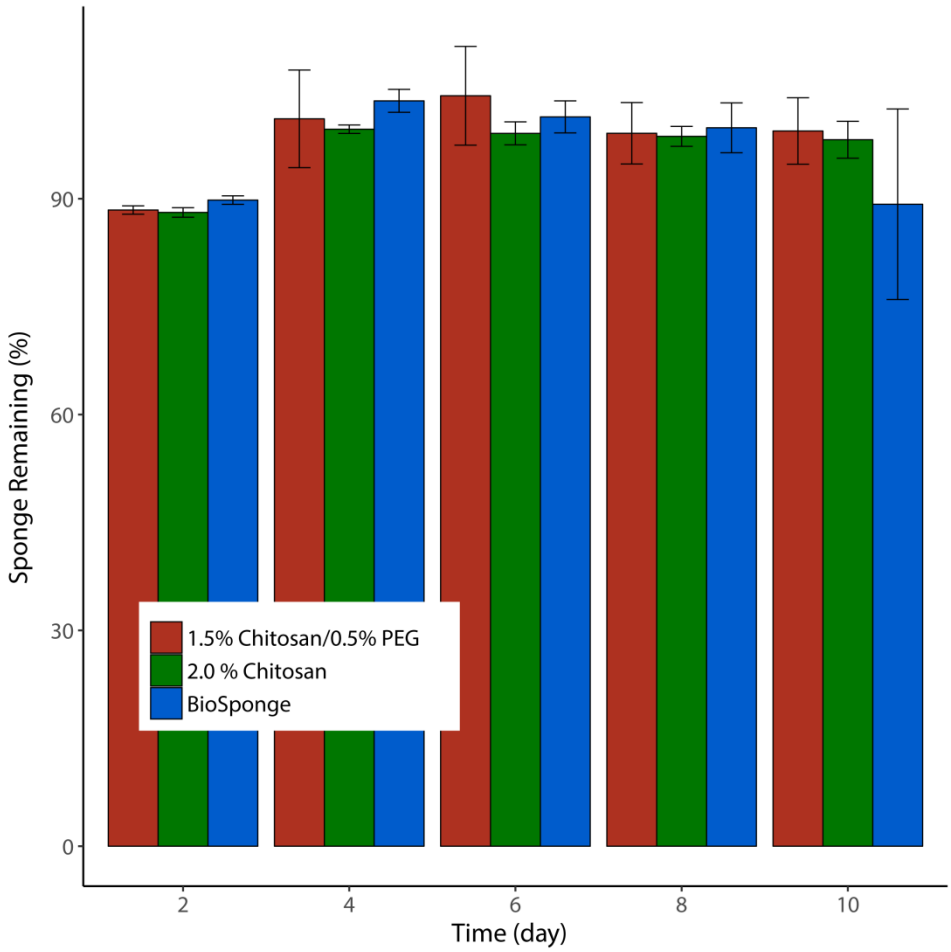

FIGURE 19. Bar graph of mean ± standard deviation of sponge remaining mass as a percentage when subjected to lysozyme mediated testing. Five samples were taken every 2 days for a duration of 10 days.

APPENDIX E: SWELLING RATIO OF CHITOSAN SPONGES

Rationale

Local delivery systems should be able to be loaded with clinician's choice of antibiotics, and accept the necessary volume to properly deliver concentrations of antibiotics above the MIC. Chitosan sponges' ability to be loaded was determined through implementation of a swelling ratio study.

Materials and Methods

Sponges were fabricated or obtained as detailed in Appendix A. Sponges (n = 4) were sectioned (6.25 cm^2) and independently weighed. Sectioned sponges were placed in 250 mL containers large enough for the section to lie flat. Sponges were hydrated with 30 mL of 1X PBS for 10 minutes. At expiration of the 10 minutes, sponges were removed with efforts taken for excess fluid to drip before loaded weight was measured. Pre- and post-hydration weights were used to determine the swelling ratio for each sponge investigated. Results can be seen in Figure 20.

Results

All sponges possess an ability to absorb at least 10 times their initial mass. The amount of chitosan appears to correlate to an increase in swelling capability.

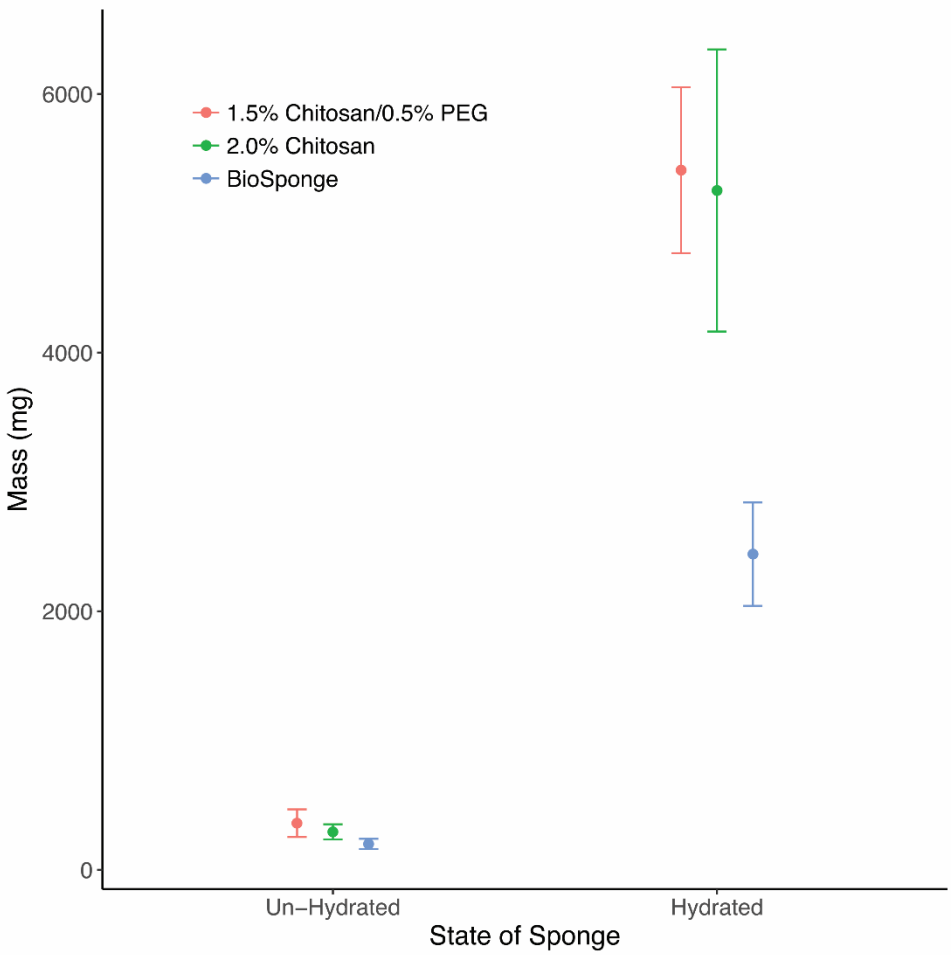

FIGURE 20. Scatter plot showing mean ± standard deviation of tested sponges (n = 5) dry and aqueous-hydrated masses.

APPENDIX F: CHITOSAN SPONGE CYTOCOMPATIBILTY

Rationale

Any successful local delivery based treatment method must be able to inhibit bacteria with minimal adverse effects. Sterile sponges were used in the cytocompatibility study phase. Once the study proved that the proposed combination therapy had good elution profiles and sponge eluates were active against bacteria cytocompatibility was investigated.

Materials and Methods

The sponges used for this phase were fabricated or obtained as detailed in Appendix A. The materials used for this experiment are: NIH3T3 cancerous fibroblasts cells, 0.75 cm^2 48-well plates, High Glucose Dulbecco's Modified Eagle Medium (DMEM) cell media, 96-well plates (Costar), Promega Cell Titer-Glo Luminescent Cell Viability assay, 70% ethanol, and chitosan sponges (n = 5). The cells were in media, which consisted of 2 mL DMEM, 10% fetal bovine serum (FBS), and 1% AB/AM. The 1% AB/AM contained penicillin, streptomycin, and amphotericin B. Cells were suspended in 6 mL of DMEM cell media and vortexed in preparation for the cytocompatibility study. NIH3T3 cells were seeded/pipetted into 48-well plates at 150µL per well and incubated overnight promoting cell attachment to the wells. Controls were tested therefore 20 wells were seeded with 150µL of cells. After 24 hours, three types of chitosan sponges (2.0% chitosan, 1.5% chitosan/0.5% PEG, and BioSponge) were added to the wells containing the cells. Each sponge was sectioned to fit inside the well plates (< 0.75 cm^2). After placing sponges in the wells, an additional 150 µL of DMEM cell media was added to each well including controls. The plates were then placed in the incubator at 37° C with 5.0% CO_2 atmosphere overnight promoting proliferation. Two plates were required due to the time points

being 24 and 72 hours. Plates were removed at their time point and sponge samples were removed, media was refreshed every 24 hours.

To perform the Cell Titer Glo luminescence assay, 50 µL of cell-titer was pipetted into each well and the plate is shaken lightly. Light shaking of the plate mixes the Cell Titer Glo with the cell media producing a bright yellow color. After the prior step, 50 µL samples were removed from each well and transferred into a white polystyrene 96-well plate, taking care to interchange the pipette tip after each well. The 96-well plate was placed in a Gen5 microplate reader and luminescence at $\lambda = 540$ nm was read with its imaging software. This is correlated with the number of cells remaining because the Cell Titer Glo mixture results in cell lysis thus generating a luminescent signal proportional to the amount of ATP (adenosine-5-triphosphate) present. The amount of ATP is directly proportional to the number of cells. In addition, imaging techniques were used to visually detect cell behavior. Healthy growing cells are viewed as spindle shaped, branching out and connected. A representative micrograph can be seen in Figure 7, which was similar for both time points and all tested sponges.

Results

Results from luminescence (Figures 21 & 22) and micrographs agree, only one micrograph displayed due to extreme similarities (Figure 20). This supports the idea that chitosan sponges are cytocompatible.

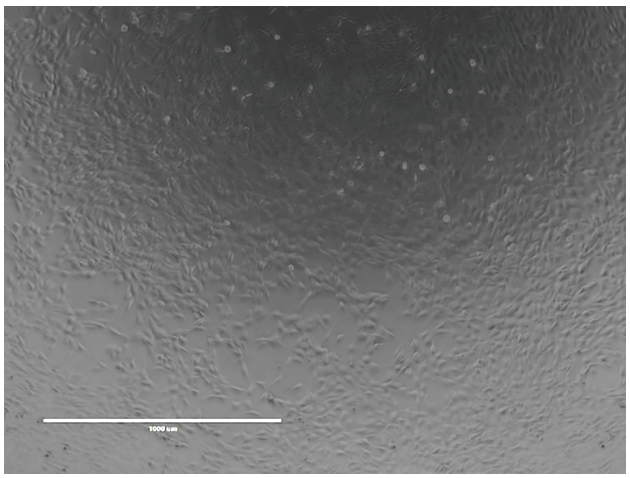

FIGURE 21. Appearance of NIH3T3 cells at 24-hours after contact with BioSponge.

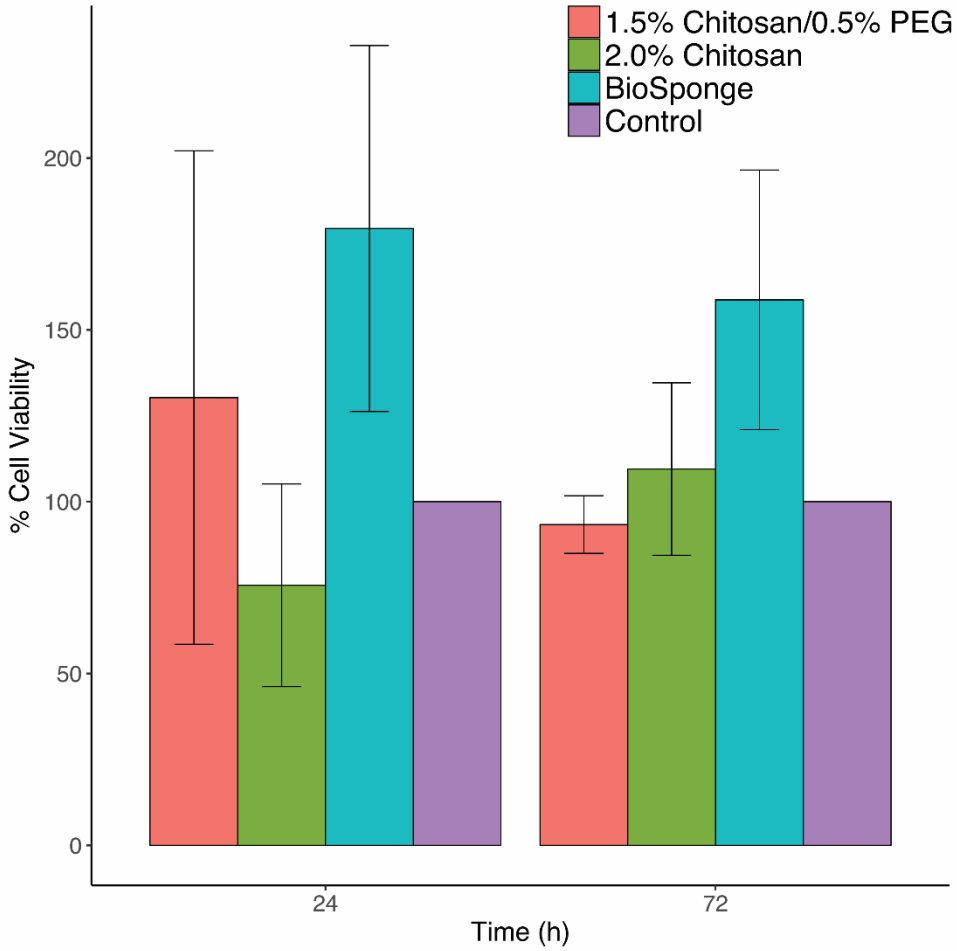

FIGURE 22. Bar graph displaying mean ± standard deviation of cell viability after being in contact with sponges (n = 5).

APPENDIX G: IACUC ANIMAL USE PROTOCOL APPROVAL LETTER

Vice Chancellor for Research

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UAMS
UNIVERSITY OF ARKANSAS
FOR MEDICAL SCIENCES

DATE: January 25, 2015

TITLE: Investigating a Flexible, Degradable Antibiotic Delivery System in Murine Infected Pin Model

AUP FILE#: 3579

PRINCIPAL INVESTIGATOR: Mark Smeltzer, Ph.D. **MAIL SLOT:** 511

NAME OF INSTITUTION: University of Arkansas for Medical Sciences

Dear Dr. Smeltzer:

The above mentioned new protocol submitted to the UAMS Institutional Animal Care and Use Committee (IACUC) was reviewed on November 21, 2014.

Revisions to the study were reviewed by the IACUC Chairman, and **Full Approval was received for the study on January 21, 2015.**

The Committee wishes to remind you that, under the provisions of the UAMS Animal Welfare Assurance Number #A3063-01 from the Office of Laboratory Animal Welfare, the principal investigator or project director is directly responsible for keeping the IACUC informed of any proposed changes involving the care and/or use of animals.

Please use the file number shown above regarding any questions or revision/addendum concerning this approved protocol.

Sincerely,

Bill J. Gurley, Ph.D.
Professor, College of Pharmacy
Chairman, Institutional Animal Care and Use Committee